Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Differential expression and functional characterization of cattle tick genes in response to pathogen infection (*Babesia bigemina*)

Sandra Isabel da Conceição Antunes

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE DE PARASITOLOGIA MÉDICA

Outubro, 2013

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Thesis: "Differential expression and functional characterization of cattle tick genes in response to pathogen infection (*Babesia bigemina*)"

Author: Sandra Isabel da Conceição Antunes

Supervisor: Investigadora Doutora Ana Isabel Amaro Gonçalves Domingos, (IHMT, UNL)

Co-supervisor: Professor Doutor Virgílio Estólio do Rosário (IHMT,UNL)
Professor Doutor José de la Fuente (IREC, UCLM)

Dissertation presented to obtain a PhD degree in Biomedical Sciences, speciality of Medical Parasitology.

Financial support was provided by Fundação para a Ciência e Tecnologia (FCT) with a PhD fellowship Grant SFRH / BD / 48251 / 2008.

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Tese: Expressão diferenciada e caracterização funcional de genes de carraças em resposta a infecção por agentes patogénicos (*Babesia bigemina*)

Autor: Sandra Isabel da Conceição Antunes

Orientador: Investigadora Doutora Ana Isabel Amaro Gonçalves Domingos (IHMT, UNL)

Co-orientador: Professor Doutor Virgílio Estólio do Rosário (IHMT,UNL)
Professor Doutor José de la Fuente (IREC, UCLM)

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Parasitologia Médica.

Apoio financeiro pela Fundação para a Ciência e Tecnologia (FCT) com a bolsa de doutoramento SFRH / BD / 48251 / 2008.

The results obtained during the development of this PhD project incited the following original research articles:

Antunes S, Galindo RC, Almazán C, Rudenko N, Golovchenko M, Grubhoffer L, Shkap V, do Rosário V, de la Fuente J, Domingos A. 2012. "Functional genomics studies of *Rhipicephalus (Boophilus) annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*." **Int J Parasitol**: 42(2):187-95

Antunes S, Merino O, Mosqueda J, Moreno-Cid JA, Bell-Sakyi L, Fragkoudis R, Weisheit S, Pérez de la Lastra JM, Alberdi P, Domingos A, de la Fuente J. 2014. "Characterization of tick proteins involved in tick-pathogen interactions as potential antigens for the control of tick infestation and pathogen infection." **Parasit Vectors**: 7: 42

Merino O, Antunes S, Mosqueda J, Moreno-Cid JA, Pérez de la Lastra JM, Rosario-Cruz R, Rodríguez S, Domingos A, de la Fuente, J. 2013. "Vaccination with proteins involved in tick-pathogen interactions reduces vector infestations and pathogen infection." **Vaccine**: 31(49):5889-5896

Dedico esta tese á minha Mãe…
Uma guerreira desconhecida pelo Mundo mas reconhecida pelos que a rodeiam!
Vou sempre acreditar na tua força!

Acknowlegments

This thesis would not have been realized without the support and collaboration from a great many people over the years and I would like to take this opportunity to thank them all. First and foremost, I would like to express my deep gratitude to Ana Domingos and Jose de la Fuente my research supervisors, for their patient guidance, enthusiastic encouragement, useful critiques that pushed me further and also for their friendship. Also I want to express my appreciation to Prof. Virgílio do Rosário for his help; whenever necessary he was always present.

When I began this long journey I already suspected that this would be a bumpy road… But what a ride it was!!! If not for anything else the experience of working with so many different people alone was so worthy. This project allowed me to meet and work with amazing persons in different places. A special thanks to Doctor Varda Shkap that kindly provided ticks, fundamental to all the work developed in this project. Also I want to express my gratitude to all the technical support in the histological cuts given by Doctor Jorge Correia and Maria do Rosário from FMV. So now: first in Ciudad Real, Ruth Galindo: thank so so much for the help and patience in the lab; I will be forever grateful for all you teach me and for your sincere friendship! Tell your Mum that I remember her often and I always smile when thinking of the time I spent with you. Mario Canales: thank you for sharing your family time with me! Jose Manuel: I´m grateful for all the advices and guidance you gave me during my stays at IREC. Juan Moreno-Cid: thanks for the friendship. To all the staff in IREC thanks, I really enjoyed working in IREC, it as a good energy!!! In České Budějovice: I´m so grateful to Doctor Libor Grubhoffer that found time in his busy schedule to take me to inspiring places, like Český Krumlov. Natasha and Marina you both are so special to me. I will forever treasure your friendship and genuine interest in my life; moreover you helped me to reach my first big goal in the PhD project so indeed you have a special place in my heart. Again to all the people in the Academy of Sciences that in some way helped me I leave here my sincere gratitude. My Mexican adventure before and after João: in Ciudad Victória a special acknowledgement to Chelo for hers professional guidance, valuable support and friendship and to Octávio my research buddy! If not for both of you I would be still looking to cows and ticks not knowing what to do, like a donkey looking to a palace!!!

Lorena, Uriel, Sarahy and Orlando you were not forgot! Thanks for the smiles and rides along the city! In my second time around in Mexico, now in Querétaro, I had the pleasure of meeting Doctor Juan Mosqueda and Gabriela and to work again with Octávio. Doctor Juan, I'm grateful for all the advices and support you gave me in this last part of the project. Octávio again thank you so much for sharing the work and your good vibes! Finally, aos meus colegas e amigos portugueses! Esta viagem começou no INETI no grupo da UTPAM onde conheci a Ana Domingos, a Bé, o Tiago, Ana Armada, Dr. Carlos, o Fernando, a Ângela, a Maria, a Raquel e a Cristina. Este grupo maravilhoso aceitou-me de braços abertos e apesar de já não existir deixa muitas saudades. No IHMT tenho de agradecer a todos, os quais me ajudaram directa ou indirectamente a terminar este projecto de doutoramento: Renato, Cátia, Tiago, Pedro, Isabel, Ana Afonso, Manuela, Prof. Paulo Almeida, Prof. Silvana Belo, obrigado pelo interesse no meu trabalho e pela amizade. À Fábia, Joana e Lara o meu sincero obrigado por partilharem comigo algum do trabalho!

Quero deixar um agradecimento especial a três pessoas que de facto foram muito importantes para mim neste percurso: Cristina, muito obrigado por seres minha amiga apesar de eu ser relaxada e esquecida por sorrires mesmo when you´re feeling down. Raquel és uma pessoa extraordinária com muita força e determinação e adorei estar grávida contigo. Ana, o apoio que me dá em todas as alturas e em todos os campos tem sido fundamental, obrigado por partilhar comigo os bons e maus momentos.

Não posso deixar de agradecer à minha família por todo o apoio: Aos meus pais, Alberto e Susete, pelos sacrifícios que fizeram e ainda fazem para me darem esta oportunidade. Ao meu irmão Hugo, pelo apoio sempre discreto. À minha irmã Zélia e ao meu cunhado Zé por acreditarem e me incentivarem a lutar pelo que quero. Claro aos meus sobrinhos adorados Sara e Pedro! Maria Inês, não me esqueci de ti, nem dos teus pais e avós! À Carina (que já é da família!) obrigado porque sim… Não podia sonhar com uma família melhor! Comparo muitas vezes a minha família a um circo onde há espaço para todos, alegria todos dias e força para enfrentar os desafios da vida!

Ao Pedro por amar os meus defeitos tal como amo os dele, por tudo e por nada, por existirmos juntos… Ao João o melhor acidente de todos os tempos, o filho mais perfeito! Que felicidade construir e partilhar a minha vida com ambos!

Resumo

Expressão diferenciada e caracterização funcional de genes de carraças em resposta a infecção por agentes patogénicos (*Babesia bigemina*)

Sandra Isabel da Conceição Antunes

Palavras-chave: Carraça, Vacina, *Babesia*, RNA de interferência, TROSPA

O conceito "*One health*" reconhece a necessidade do trabalho conjunto de veterinários, profissionais de saúde e cientistas, dada a interface dinâmica entre pessoas, animais e ambiente. Este conceito é muito importante em zoonoses, tais como doenças associadas a carraças (DAC´s) que dependem de animais como reservatório. Os protozoários do género *Babesia* são agentes patogénicos transmitidos por carraças que causam a doença denominada babesiose num variado número de animais incluindo o Homem. Particularmente a *B. bovis* e *B. bigemina* são transmitidas por carraças, relacionadas com gado, *Rhipicephalus (Boophilus) annulatus* e *R. microplus* sendo estas consideradas os ectoparasitas de maior importância, com largo impacto económico na produção animal. O uso tradicional de acaricidas no controlo de carraças apresenta diversas desvantagens incluindo a seleção de carraças acaricido-resistentes e contaminação ambiental com resíduos químicos. As vacinas destacam-se como uma alternativa ao uso de acaricidas. O objetivo destas vacinas é a proteção contra DAC´s através do controlo das infestações pelos vectores e redução de transmissão de doença. As proteínas envolvidas nas interações carraça-agente patogénico podem ser bons candidatos para essas novas vacinas mas a sua identificação e validação continuam a ser obstáculos. Os objetivos do presente estudo foram, primeiro, a identificação de genes de *R. annulatus* diferenciadamente expressos em resposta à infeção por *B. bigemina*, segundo, a validação da influência destes genes no processo de infeção e finalmente a caracterização dos antigénios identificados, a fim de selecionar os melhores candidatos, para o desenvolvimento de uma potencial nova vacina. A fim de alcançar os objectivos propostos, clones de uma biblioteca de hibridização subtrativa por supressão (SSH) foram sequenciados e analisados. Os genes diferenciadamente expressos com prováveis funções relacionadas com a interface carraça- agente patogénico, foram selecionadas para validação dos resultados de SSH por real time RT-PCR. A análise funcional conduzida por RNA de interferência mostra que, nas condições do presente estudo, o silenciamento dos genes que codificam para as proteínas, sérica amilóide A e TROSPA levam á redução de níveis de infecção em *R. annulatus* e em *R. microplus* em comparação com o grupo controlo. Em *R. microplus* é demonstrada a influência também da calreticulina (CRT). As proteínas TROSPA e CRT foram selecionadas e obtidas usando um sistema de expressão em *Escherichia coli* e anticorpos poli/monoclonais foram produzidos. O reconhecimento das proteínas nativas foi confirmado por Western blotting e imunofluorescência em tecidos de carraça. O efeito dos anticorpos específicos, suplementados à refeição de sangue, foi demonstrado pela avaliação do peso final e/ou ovoposição em carraças alimentadas artificialmente. Não foi observado efeito significativo na aquisição de *B. bigemina*. Os resultados mostram as vantagens e desvantagens do sistema *in vitro* de alimentação artificial de carraças por tubos capilares na caracterização de antigénios protetores de carraça. Diferentes estudos caracterizaram a interface carraça- agente patogénico a nível molecular no entanto, o presente estudo apresenta a primeira análise funcional de genes em carraças infectadas com *B. bigemina*. Os resultados apresentados contribuem para um maior conhecimento do papel de genes de carraça no processo de infeção/multiplicação por *Babesia* sp., bem como para o desenvolvimento de novas vacinas.

Abstract

Differential expression and functional characterization of cattle tick genes in response to pathogen infection (*Babesia bigemina*)

Sandra Isabel da Conceição Antunes

Keywords: Tick, Vaccine, *Babesia*, RNA interference, TROSPA

The "*One Health*" approach recognizes the need for veterinarians, human health professionals, and environmental scientists to work together given the dynamic interface among people, animals, and the environment. This approach is increasingly important for zoonotic diseases, such as tick borne diseases (TBD´s) which rely on animals as reservoirs. *Babesia* spp. are tick-borne pathogens that cause a disease called babesiosis in a wide range of animals and also humans. Particularity, *B. bovis* and *B. bigemina* are transmitted by cattle ticks, *Rhipicephalus (Boophilus) annulatus* and *R. microplus* being considered the most important cattle ectoparasites with major economic impact on cattle production. The traditional use of chemicals to control ticks has serious drawbacks, including the selection of acaricide-resistant ticks and contamination of the environment with chemical residues. Research on alternatives to the use of acaricides is strongly represented by tick vaccines considered a more cost-effective and environmentally safe strategy. The ultimate goal of tick vaccines is to protect against TBD´s through the control of vector infestations and reducing pathogen infection and transmission. Tick proteins involved in tick-pathogen interactions may provide good candidate protective antigens for these new vaccines but their identification and validation are still limiting steps. The objectives of this study were first to identify *R. annulatus* genes differentially expressed in response to infection with *B. bigemina*, second to validate the influence of these genes in the infection in both *R. annulatus* and *R. microplus* ticks and finally to characterize identified antigens to select the best candidates for future vaccine development. In order to achieve these goals, suppression-subtractive hybridization (SSH) library clones were sequenced and analyzed. After molecular function gene ontology assignment differentially expressed genes with putative functions in tick-pathogen interactions were selected for validation of SSH results by real-time RT-PCR. Functional analysis by RNA interference showed that under the conditions of the present study, knockdown of *trospa* and *serum amyloid A* significantly reduced *B. bigemina* infection levels in *R. annulatus* and *R. microplus* when compared to controls. In *R. microplus* also calreticulin showed infection reduction. TROSPA and CRT were selected, recombinant proteins were obtained using *Escherichia coli* expression system and poly/monoclonal antibodies were generated. Their specificity against tick recombinant proteins was confirmed by Western blotting and against native proteins in tick tissues using immunofluorescence. Capillary-fed ticks ingested antibodies added to the blood meal and the effect of these antibodies on tick weight and/or oviposition was shown. No significant effect was observed on pathogen acquisition. The results highlighted the advantages and disadvantages of *in vitro* tick capillary feeding for the characterization of candidate tick protective antigens. Several studies have characterized the tick-pathogen interface at the molecular level. However, to our knowledge this is the first report of functional genomics studies in ticks infected with *B. bigemina*. The results reported here increased our understanding of the role of tick genes in *Babesia* infection/multiplication and contribute to the development of a vaccine with impact on both tick infestation and pathogen infection.

Abbreviations

µg	Microgram
µl	Microliter
µm	Micrometer
ºC	Degree Celsius
64TRP	64 tick recombinant protein
Abs	Absorbance
A.D.	Anno Domini
AKR	Akirin
AMA-1	Apical membrane antigen 1
Amp	Ampicillin
AP	Alkaline Phosphatase
B.C.	Before Christ
Bbo-MIC1	Recombinant microneme protein from *Babesia bovis*
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CRT	Calreticulin
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dNTPs	Deoxynucleotide triphosphates
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DAPI	4,6-diamidino-2-phenylindole
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DNA	Desoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
e.g.	*exempli gratia*
ELI	Expression library immunization

ELISA	Enzyme-linked immunoabsorbent assay
ER	Endoplasmatic reticulum
EST	Expressed sequence tag
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
G	Gauge
g	G-force
GO	Gene ontology
HAT	Hypoxanthine-aminopterin-thymidine
HAZV	Hazara vírus
HGPRT	Hypoxanthine guanidine phosphoribosyltransferase
His	Histidine
i.e.	*id est*
IFA	Indirect fluorescent antibody assay
IgGs	Imunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilo base
kDa	Kilo Dalton
kHz	Kilo Hertz
KTPI	Kunitz-type protease inhibitor
LB	Luria Bertani
M	Molar
mA	Milliampere
Ml	Millilitre
min	Minute
mM	Millimolar
Mab´s	Monoclonal antibodies
mRNA	Messenger RNA
nm	Nanometer
OD	Optical density
ospA	Outer surface protein A
ospC	Outer surface protein C

OTEs	Off-target effects
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVC	Packed cell volume
PVDF	Polyvinylidene fluoride
RAS-3	*Rhipicephalus appendiculatus* serpin 3
RAS-4	*Rhipicephalus appendiculatus* serpin 4
RBC	Red blood cells
rCRT	Recombinant calreticulin
rDNA	Ribossomal DNA
RIM36	Immuno-dominant protein of *Rhipicephalus appendiculatus*
rTROSPA	Recombinant TROSPA
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Rotations per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNAs	Small interfering RNAs
TAE	Tris acetate buffer
TBD	Tick borne diseases
TBE	Tick borne encephalitis
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
Tris-HCl	Tris hydrochloride
TROSPA	Tick receptor for outer surface protein A
TTBS	Tween-Tris buffer saline
RNAseq	RNA sequencing
s	Second
s.s.	*sensu stricto*
s.l.	*sensu lato*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

spp.	Species (plural)
SSH	Suppression-Subtractive Hybridization
S.O.C.	Super-optimal catabolite repression médium
SUB	Subolesin
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WB	Western blotting

Table of contents

Index of figures

Index of tables

Introduction

1.1 Ticks

Ticks have evolved to become one of the most important groups of arthropod vectors of pathogens. Found in most terrestrial regions of the earth. Ticks are a highly specialized group of obligate, bloodsucking, nonpermanent ectoparasitic arthropods that feed on mammals, birds, and reptiles (Schwan, 2011). These haematophagous ectoparasites inflict a direct negative impact on the vertebrate hosts while they feed and most importantly they may also act as vectors, as well as reservoirs, of multiple pathogens (Jongejan and Uilenberg, 2004). While considered zoophilic, many species are associated to the transmission of important ethological agents to humans (Silva et al., 2006). To qualify as a vector, a tick must (1) feed on infectious vertebrates (2) acquire the pathogen during the blood meal (3) maintain the pathogen through one or more life stages (4) transmit the pathogen to previously unexposed hosts while feeding again (Kahl et al., 2002, Jongejan and Uilenberg, 2004, Estrada-Pena et al., 2013).

1.2 Historical overview

Fossil records suggest that ticks originated 65–146 million years ago in the Cretaceous period from the Mesozoic Era (Klompen and Grimaldi, 2001, Nava et al., 2009).

Figure 1: Drawing of hyena-like animal showing tick infestation.
Discovered in the Egyptian tomb of Antef, dating from the time of Thutmose III (eighteenth dynasty), about 1500 B.C. *(from Nijhof, 2010)*.

A reference to - what could be - 'tick fever' was found on a papyrus scroll dating back to the 16th Century B.C. (Krantz, 1978, Heyman et al., 2010), and an animal drawing dating back to the Queen Hatshepsut III era (15th Century B.C) (Figure 1) shows what were considered to be three ticks attached to a hyena's ear (Arthur, 1965, Heyman et al., 2010).The Greek philosopher Aristotle (384-322 B.C.) wrote in his "*Historia Animalium"* "The tick is generated from couch-grass" and that "cattle suffer both from lice and from ticks. Sheep and goats breed ticks, but do not breed lice" (Aristotle, 350 B.C.). In an early encyclopedia *"Historia Naturalis",* published A.D.77–79 by Pliny the Elder, ticks are described: 'a tick simply filled to bursting point with its victim's blood and then died because it had no anus' (Heyman et al., 2010, Nijhof, 2010). It is obvious that the hostile nature of ticks was long ago recognized.

1.3 Characterization, identification, morphology

1.3.1 Classification

Ticks belong to phylum Arthropoda, subphylum Chelicerata, class Arachnida, sharing the subclass Acari together with mites, subclass Acari, superorder Parasitiformes, order Ixodida and superfamily Ixodoidea. Approximately 879 different tick species have been described to date (Nava et al., 2009). Ticks are further divided into three families: Argasidae or soft ticks with 185 species, Ixodidae or hard ticks with 713 species and Nuttalliellidae with only one species (Barker and Murrell, 2004). A hard sclerotized shield or scutum, found on the anterior dorsal surface of hard ticks, is absent in soft ticks and forms the most remarkable difference between these two families. Other anatomical differences include the aspect of the outer body wall or integument which is leathery in soft ticks while smooth with fine grooves in hard ticks, and the position of the mouthparts. These are located ventrally in soft ticks and anterior in hard ticks, making them always visible from a dorsal aspect in ticks of the latter family. The sole representative of Nuttalliellidae family is *Nuttalliella namaqua* Bedford, 1931, found in Southern Africa from Tanzania to Namibia (Aragão, 1936, Latif et al., 2012) and it is considered the most basal lineage of ticks with features characteristic of both soft and hard ticks (Elshoura, 1990, Klompen et al., 2007, Mans et al., 2011). The hard ticks form the largest family and can be divided in two lines: the Prostriata and the Metastriata. The

Prostriata is regarded as the most primitive line and consist of the *Ixodes* genus only, which can copulate both on and off the host in contrast to Metastriata adults which mate only on the host (Barker and Murrell, 2004). Examples of hard and soft ticks are represented in figure 2.

Figure 2: Tick examples.
(A) dorsal (left) and ventral (right) view of a *Rhipicehalus annulatus* female, representative of a hard tick species (*original from the author*). (B) dorsal (left) and ventral (right) view of an *Ornithodoros savignyi* Audouin, 1827, female, representative of a soft tick species (*original and authorized from Ard Nijhof*).

1.3.2 Life cycle

Ticks undergo four stages, namely egg, larvae, nymph and adult (Oliver, 1989, Sonenshine, 1991). Ixodid have only one nymph instar, in contrast to the several nymphal instars of argasid ticks (Oliver, 1989). Ixodid ticks need several days to feed, and once the female is engorged she drops from the host to deposit thousands of eggs and dies. Argasid ticks feed more than once and intermittently and these parasites don´t remain attached to their hosts. These ticks may feed several times during their lifetime and on a number of different hosts laying few hundred eggs in batches. Argasid ticks have a remarkable longevity living for many years and may endure long period of starvation (Sonenshine, 1991). Longevity in fact is remarkable in argasid ticks: there are records of *Ornithodoros canestrinii* Clifford, Kohls & Sonenshine, 1964, survival in laboratory of 10 years without food and impressively an unfed adult of *O. lahorensis* Neumann, 1908, for 18 years (Hoogstraal, 1985, Oliver, 1989). The most distinctive features between soft and hard ticks are summarized in table 1.

Table 1: Main features that distinguish Ixodid and Argasid ticks (*adapted from University, 2013)*

Features	Hard Ticks	Soft Ticks
Scutum (dorsal shield)	Present	Not presente
Capitulum anterior (mouthparts)	Visible from dorsal view	Not visible from the dorsal view
Nymphal stages	One	Several
Adult feeding time	Days	30-60 min
Female blood meals	One	Several
Oviposition	One	Several
Total eggs laid	3.000-8.000	400-500

In Ixodid ticks larval, nymphal and adult feeding usually requires 3-7, 4-8 and 7-9 days respectively, during which time active growth of gut and cuticle occurs in order to accommodate the blood meal, most of which will be acquired in the final 24 hours of engorgement. Male Ixodids do not feed as females. They feed intermittently, small quantities of blood and enough for their reproductive organs to mature. Male ticks in the genus *Ixodes* have active reproductive organs as soon as they moult from the nymphal stage and do not need to feed. In nature, tick life cycles are regulated by seasons. The length of life cycles is quite variable in result to several constrains such as photoperiods, temperature, humidity and availability of suitable hosts. *Rhipicephalus microplus* Canestrini, 1887, ticks can ensure three or four generations a year: one generation a year is probably the usual pattern for most of the ticks in the subtropics and warmer regions. But in colder regions, ticks can take one to three years to complete their life cycle protracting it until better conditions arise (Oliver, 1989, Sonenshine, 1991).

1.3.3 Tick-host interactions

Ixodid ticks can be three-, two-, or one-host arthropods. For three-host ticks, larvae feed on a host, fall off, and molt into a nymph. The nymph then attaches to another host, feeds, and falls off, and finally the adult attaches to a third host and feeds (Figure 3B). In the two host ticks, larvae attach to the host and upon repletion they hatch and

nymphs reattach feeding again until repletion. After nymphs drop from the host and after some days (or longer) adults hatch and seek a new host to complete the life cycle. One-host ticks attach to a host as larvae, and then feed and mature to the adult stage on the same host (Schwan, 2011) (Figure 3A).

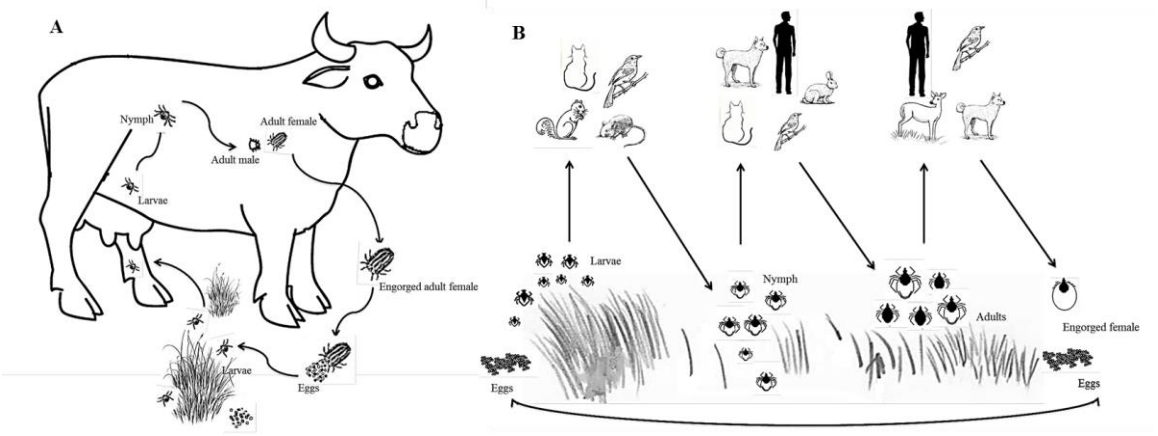

Figure 3: Characteristic Ixodid life cycles.
A) The one host life cycle. B) Three-host life cyle *(original from the author)*.

There is some flexibility in this feeding behavior and under certain conditions ticks can use one or two hosts (*e.g. Hyalomma scupense*, Schulze, 1919) or use two instead of three (*e.g. Amblyomma rotundatum*, Koch 1844) (Oliver, 1989). Some ticks accept a wide variety of host species, other might be more selective and some others are extremely demanding and attach to only one host species. In fact, with the exception of fish, ticks parasite all vertebrates (mammals, birds, reptiles and amphibians). Nevertheless, mammals are the dominant host for most of Ixodid ticks.

Ticks are adapted to two contrasting components of their habitat: the physical environment and their host. The most important component of the physical habitat of a tick is the climate that is defined by temperature and humidity. When ticks are moulting and then questing in the physical habitat they are in danger of drying out, starving and they are also exposed to predators such as rodents, birds, reptiles, ants and to pathogens such as fungi (Latif and Walker, 2004). High temperatures and body-water homeostasis are of great importance in processes that influence off-host survival. Ticks as a group have the capacity to survive without food and /or water longer than most other arthropods (Needham and Teel, 1991). These off-host periods are characterized by slow metabolism

with long intervals of immobility which can be interrupted when water uptake can be established or to seek for a host. When on the host, there is no longer danger of drying out or starving, but they can be removed by host's grooming or feeding might be reduced by host immunity. Most ticks have adaptations in their behavior (*e.g.* attachment site) and physiology of feeding thus reducing host reactions. Host-acquired immunity might be expressed in various ways ranging from rejection of the parasite to increasing feeding time, inadequate engorgement leading to decreased egg viability or infertility (Parizi et al., 2012, Willadsen and Jongejan, 1999).

1.3.4 Overview of tick anatomy and physiology

Externally ticks have bodies which are divided into two primary parts: the anterior capitulum (or gnathosoma) containing the head and mouthparts; and the posterior idiosoma which contains the legs, digestive tract, and reproductive organs. The idiosoma is distinguished to podosoma with chelicerae, palps, hypostome and four pairs of legs in adults, and opisthosoma with anal aperture. The hypostome is modified specifically for blood-feeding and is essential for the attachment to the host. Chemosensillas, mechanosensillas and eyes or photoreceptors are located on the capitulum. Ticks also have a special sensory organ - Haller's organ – on the tarsus of the first pair of legs that provides information about host location, host odors, or detection of pheromones. Cuticle covers whole tick body and serves as an exoskeleton (like in other arthropods). A layer of cuticle called procuticle, specifically its outer part, becomes sclerotized in certain parts and forms sclerites. The biggest sclerite, scutum, covers the anterior part of the body and protects the dorsal side of it. The major components of the cuticle are proteins and chitin; lipids represent a minor part (Sonenshine, 1991).

Internally, ticks possess different organs surrounded by hemolymph. Since most experiments in ticks are traditionally focused on the organs that can be easily dissected from the feeding tick female (Figure 4) - the midgut, salivary glands and the ovary, those are the ones described here.

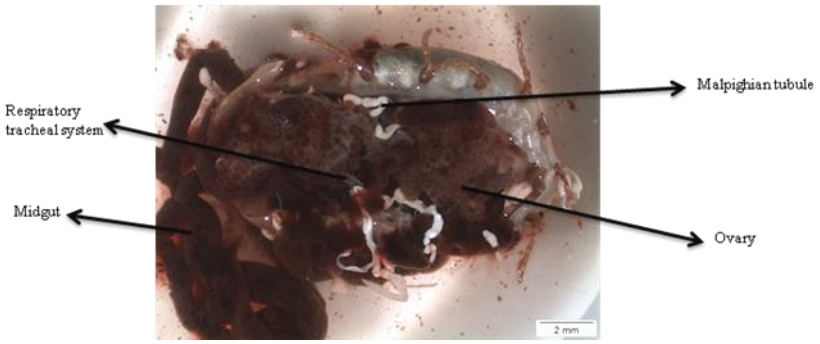

Figure 4: Dissection of an engorged *Rhipicephalus annulatus* female tick.
After removal of part of the cuticle midgut, ovary, malpighian tubules and tracheal system are effortlessly observed. *(original from the author).*

The midgut is the most prominent organ in the tick body. According to Coons and Alberti (1999), the midgut of ticks is divided into an anterior and a post-ventricular region, lined by a simple pseudo-stratified epithelium composed of cells that have distinct classifications and functions. In general, six different cell types have been described in the anterior midgut of many tick species, namely, replacement (or stem cells), digestive, secretory, undifferentiated, endocrine, and vitelogenic cells (Remedio et al., 2013). During feeding, it occupies almost the whole body cavity of the tick. The midgut branches serve as storage place. Contrary to insect, the digestion in ticks is an intracellular process (with the exception of the intraluminal digestion of erythrocytes) (Sonenshine, 1991, Coons and Alberti, 1999). Ultra structurally, the cells of the midgut of Ixodidae ticks in general are complex, having different organelles and many cytoplasmic inclusions thus reflecting the multifunctional activities of the midgut (Caperucci et al., 2010).

Salivary glands (Figure 5) perform a variety of complex functions important to tick survival as well as for the development of tick-borne pathogens (Sonenshine, 1991). In both Argasid and Ixodid ticks the pair of salivary glands is located in the lateral regions of the body cavity, described as grape-like (alveolar structures) clusters composed of the granular and agranular acini. A system of small secondary ducts drains saliva to the main duct towards the opening in the mouthpart (Sonenshine, 1991). Morphological studies have revealed significant differences in granular acini of hard and soft ticks (Coons and Alberti, 1999). In hard ticks different types of acini (agranular type I and granular type II to IV) are described with different roles *e.g.* type I has been associated to off-host osmoregulation and type II and III are involved in the synthesis and

secretion of protein factors and water transport (Sonenshine, 1991). Before feeding salivary glands are essential in water balance regulation, during attachment and feeding are responsible for cement proteins secretion as well as other molecules transported by saliva (Sonenshine, 1991). During feeding salivary glands enlarg several times and once females fully engorge undergo degeneration and remodeling processes that are likely under hormonal regulation (Sauer et al., 2000, L'Amoreaux et al., 2003).

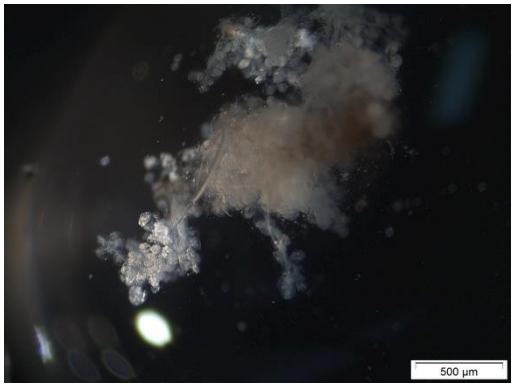

Figure 5: Dissected salivary glands from *Rhipicephalus annulatus* engorged female.
(original from the author).

Female reproductive system consists of a single U-shaped ovary: the paired oviducts, a single uterus, the vagina subdivided into cervical and vestibular regions and the seminal receptacle (organ not present in Argasid and postriate females whereas uterus is enlarged distally where it joins the vagina). Ovary is located in the posterior region of the body. In the unfed females the ovary is rather thin and small but in fed females it´s a large organ with a tube-like structure of luminal epithelium and developing oocytes connected with an epithelium by a short hollow stalk called funiculus (Figure 6) (Sonenshine, 1991). The tick oocytes remain stationary during formation and yolk deposition takes place *in situ*. The whole ovary with egg cells is expanded into the hemolymph and surrounded by several layers of acellular tunica propria. The mature oocytes (still primary oocytes without division) ovulate into the ovarian lumen. In ticks the precise site for fertilization as well as the mechanism by which male gametes encounter oocytes is still unclear but it´s generally accepted that it occurs prior to oviposition. After fertilization, the binucleate oocyte undergoes first meiotic division, but oogenesis is not completed until the oviposition, when the nuclei fuse. Embryogenesis thus occur external to the female body (Sonenshine, 1991, Saito et al., 2005).

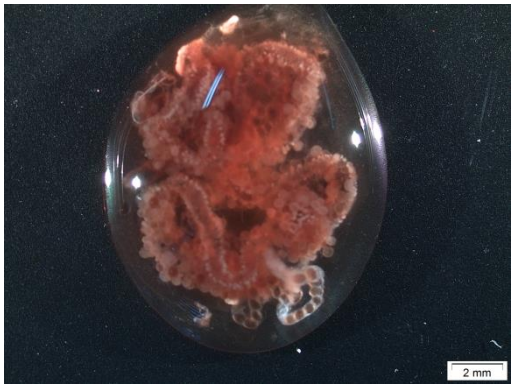

Figure 6: Dissected ovary from an engorged Rhipicephalus annulatus female tick.
(original from the author).

1.3.5 From *Boophilus* to *Rhipicephalus*

Progress in the phylogeny and evolution of ticks, in particular of hard ticks, culminates into the adjustment of nomenclature of ticks.

The genus *Boophilus* is no longer valid and has recently became part of the *Rhipicephalus* genera since there is considerable evidence from morphology and nucleotide sequences that *Rhipicephalus* is paraphyletic with respect to *Boophilus* (Murrell et al., 2000, Beati & Keirans, 2001, Murrell and Barker, 2003). To preserve the name, since it is very well known, the genus *Boophilus* has become a subgenus of the genus *Rhipicephalus*. In fact, many times these ticks are still referred as only *Boophilus* disregarding the current nomenclature. In this dissertation *Boophilus* may be used when referring *R. annulatus* and/or *R. microplus* in order to simplify the allusion to these ticks. There are five valid species of ticks in subgenus *Boophilus*: *Rhipicephalus (Boophilus) annulatus* Say, 1821, *R. decoloratus* Koch, 1844, *R. geigyi* Aeschlimann & Morel, 1965, *R. kohlsi* Hoogstraal & Kaiser, 1960 and *R. microplus* Canestrini, 1888. These ticks, commonly known as cattle ticks, present a characteristic morphology and one-host life-cycle: preferably cattle (except for *R. kohlsi* with a predilection for small ruminants), taking about three weeks to complete their cycles on the host from unfed larvae to engorged female (Jongejan and Uilenberg, 2004).

As *Rhipicephalus* (*Boophilus)* ticks are one-host ticks, they may become very numerous on cattle herds, particularly those with a low degree of resistance, and cause

considerable direct damage. Although these ticks have short mouthparts, damage to hides is considerable as the preferred feeding sites are often of good leather potential (Jongejan and Uilenberg, 2004). In addition to *R. microplus*, species of most importance to farm animals are *R. annulatus* which is widespread in tropical and sub-tropical countries and *R. decoloratus* which occurs in Africa. These species are involved in the transmission of several pathogens which escalate their importance in animal industry. These cattle ticks, are associated with the transmission of pathogens that cause babesiosis (*Babesia bovis* Babes, 1888 and *Babesia bigemina* Smith & Kilborne, 1893) and anaplasmosis (*Anaplasma marginale* Theiler, 1910) (Peter et al., 2009) which are regarded as very important diseases in cattle, leading to large economic losses (Bock et al., 2004, Shkap et al., 2007a, Suarez and Noh, 2011).

1.4 Tick control

Tick control is essential to reduce impact on livestock productivity and also to contract tick-borne diseases prevalence. Tick control is mainly based on the application of acaricides, despite their disadvantages and limitations, although other control methods such as vaccination or biocontrol agents are available (Willadsen, 2006).

1.4.1 Biological tick control

Ticks have relatively few natural enemies, but the use of predators, parasites, and pathogens has been examined towards tick control. Most predators are non-specific, opportunistic feeders and probably have little impact on ticks. Sub-Saharan African birds such as the yellow and red-billed oxpeckers (*Buphagus africanus* Linnaeus, 1766 and *Buphagus erythrorhynchus* Stanley, 1814) can eat 14 grams of ticks per day. Other general predators that can occasionally feed or parasite ticks (*e.g.* parasitoid wasp, *Ixodiphagus hookeri* Howard, 1907) may contribute to a reduction of natural tick populations, include several species of ants, beetles, flies and spiders (Jemal and Hugh-Jones, 1993, Hu and Hyland, 1998, Samish and Rehacek, 1999, Miranda-Miranda et al., 2011). Exploitation of these generalist predators is possible but has the potential danger in causing unwanted changes in the populations of non-target species.

Ticks can harbor a wide range of endosymbiotic bacteria including *Rickettsia* da Rocha-Lima, 1916, *Francisella* Dorofe'ev 1947, *Coxiella* Philip, 1948, and *Arsenophonus* Werren, 2005, amongst others (Alberdi et al., 2012).Tick control strategies could be planned based on interference with their endosymbionts for the control of the vectors and the pathogens they hold. Since these endosymbionts are essential for the subsistence of the arthropod host, their elimination would be deleterious for their survival (Ghosh et al., 2007).

The application of entomopathogenic fungi is a promising approach for controlling ticks. Of the approximately twenty species of entomopathogenic fungi which have been reported to attack and kill ticks, only a handful have been extensively studied, in particular *Metarhizium anisopliae* Sorokin, 1883 and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Kaaya et al., 1996, Pirali-Kheirabadi et al., 2007, Quinelato et al., 2012, Monteiro et al., 2013). Entomopathogenic fungi for the control of ticks have been applied in field trials both on- and off-host with reasonable success and commercial products have been developed (Samish et al., 2004, Stafford and Allan, 2010). Biocontrol agents usually favor both human and environmental safety, especially in comparison to the use of acaricides, but few products have been used in spite of their potential. The inability to successfully adopt biocontrol strategies includes factors like environmental stability (*e.g.* UV resistance, temperature tolerance), ability to initiate infection at low humidity and potential unspecific damage to non-target invertebrates (Domingos et al., 2013).

1.4.2 Genetic tick control

The genetic resistance of cattle to ticks and tick-borne diseases is complex, but, facing other control methods and their problems, breeds resistance has become an important parameter in some regions. This resistance trait is most commonly manifested in indigenous zebu (*Bos indicus* Linnaeus, 1758) breeds. The resistance status can be improved by selection of increased tick resistance as demonstrated by the development of highly tick resistant breeds such as the Belmont Adaptaur or Droughtmaster (Frisch, 1999).

Genetic control of a different order is the suggested release of sterilized ticks into the environment, similar to sterile insect techniques developed for the control of insect pests. Ticks can be sterilized through hybridization (Hilburn et al., 1991), treatment with chemicals (Hayes and Oliver, 1981) or by RNA interference (RNAi) (de la Fuente et al., 2006c, Merino et al., 2011a). Practical difficulties such as the mass rearing of sufficient amounts of sterile ticks and expected difficulties in obtaining the public and political support for the mass release of ticks may pose too high hurdles to make the field application of this technique possible.

1.4.3 Chemical tick control - Acaricides

So far, the use of acaricides has been a major component of an integrated tick control measures. Even before Smith & Kilborne (1893) proved the role of ticks in transmission of *Babesia* spp., animal health authorities in the USA, Australia and Southern Africa were treating cattle with a variety of chemical agents, mainly mixtures of querosene, sulphur and lard, in an effort to control ticks (George et al., 2004). There are many acaricides that can be used against ticks: pyrethroids as flumethrin and deltamethrin; organochlorines, as dichlorodiphenyltrichloroethane (DDT); organophosphates, as diazinon and coumaphos; carbamates, as carbaril; formamidines, as amitraz; cicloamidines as, clenpirin and macrocyclic lactones (avermectins and milbemycins), among others (George et al., 2004, Latif and Walker, 2004). Some are still available other were withdrawn from the market due limitations like contamination problems, ineffective issues or resistance arising. Acaricides endorse residual effects in milk and meat products, as well as in the environment. Schemes of acaricides application are often difficult to maintain and therefore, tend to be inappropriately used, being responsible for the increase of acaricide-resistant ticks (George et al., 2004, Graf et al., 2004). Resistance is associated with mutations in genes related to drug susceptibility, like detoxificating enzymes, as esterases, glutathione-S-transferases and mono-oxidases, and due to genetic drift (Rosario-Cruz et al., 2009, Guerrero et al., 2012a). The appearance of acaricide resistance leads to the rise of ticks individual for which the lethal dose is higher than the one for the majority of determined species. The speed, with which resistance appears, along with the significantly more expensive pesticides, has restrained the companies to develop new drugs. Combinations of various acaricides are used

worldwide; products combining active components are available in an attempt to exploit a diverse number of mechanisms of action, to reduce the emergence of insecticide resistance (Veiga et al., 2012).

The introduction of a new product in the market is time-consuming and has a huge economic burden (Graf et al., 2004). The public awareness of the detrimental effects of pesticides on the environment and increasing concerns about resistance of insecticidal compounds, demands the necessity of finding new approaches in tick control.

1.4.4 Immunological tick control - Vaccines

Major alternatives to conventional acaricide treatments have been developed in recent years and anti-tick vaccines are among the most important developments. William Trager demonstrated in 1939 that injection of guinea pigs with extracts of whole larvae, salivary glands or digestive tracts of female *Dermacentor variabilis* Say, 1821 ticks conferred partial protection against subsequent tick challenge. This partial resistance could be transferred through serum from immune animals to susceptible ones (Trager, 1939, Allen and Humphreys, 1979, Johnston et al., 1986). Immunization with heterogeneous preparations such as tissue extracts results in a polyclonal and multifactorial immune response by the experimental animal. This kind of immunization masks the antigen(s) that triggered the effective response so it became necessary to simplify mixtures injected in the hosts. Increasingly simpler midgut protein combinations were tested until an antigen named **Bm86**, which conferred significant protection of cattle against *R. microplus* infestations, was discovered (Willadsen et al., 1989). This protein of unknown function in tick biolog is localized on the microvilli of the midgut digest cells, tick ingestion of antigen specific antibodies leads to lysis of these cells resulting in mortality and a deleterious effect on the reproductive performance of tick (Kemp et al., 1989, Rodriguez et al., 1995, de la Fuente et al., 1998, Willadsen, 2004,). The discovery of this protective antigen was a milestone in the development of anti-tick vaccines. In the early 1990´s two commercial vaccines emerged containing the Bm86 recombinant protein, Gavac in Cuba and TICKGARD in Australia, TickGARD Plus (improved vaccine, contained the recombinant Bm86 antigen and BM91with a different adjuvant) was released later (Willadsen, 2004, Ghosh et al., 2007). In Australia vaccination with

TickGARD of a dairy herd resulted in a 56% reduction of tick numbers in the field over a single generation, a 72% reduction in tick reproductive performance and an increase in cattle live weight gain of 18.6 kg over a 6 month period compared to an unvaccinated control group (Jonsson et al., 2000). In Cuba, within a different context, vaccine based on the same antigen was marketed as Gavac (de la Fuente et al., 2007a, Merino et al., 2013). A retrospective analysis of the field application of Gavac from 1995 to 2003 during which almost 600,000 dairy cattle were vaccinated showed that the number of acaricide treatments was reduced by 87% with an overall reduction of 82% in the country's acaricide consumption for tick control. The incidence of mortality due to bovine babesiosis was reduced as well (Valle et al., 2004). Despite the effectiveness of these commercial Bm86-based vaccines for the control of cattle tick infestations, they show strain-to strain variation in efficacy being effective mainly against *Rhipicephalus* tick species (de la Fuente and Kocan, 2003, Willadsen, 2006, Guerrero et al., 2012b) and since Bm86 protein was found in the midgut of ticks and therefore designated 'concealed' antigen, *i.e.* not exposed to the host's immune system, regular booster is required to maintain a strong antibody response which poses a problem to convince farmers to adopt this control approach (Willadsen, 2004, Kiss et al., 2012).

These anti-tick vaccines use recombinant proteins as antigens to immunize animals and are an attractive alternative for the control of tick infestations, since they present several advantages, such as prevention or reduction of pathogens transmission (de la Fuente et al., 1998, Almazan et al., 2005, de la Fuente et al., 2007a, de la Fuente et al., 2011, Merino et al., 2011b) environmental safety, low cost production (Odongo et al., 2007, Kiss et al., 2012), prevention of drug-resistant ticks selection (Parizi et al., 2012) and inclusion of multiple antigens that could target several tick species (de la Fuente et al., 2000, de Vos et al., 2001, Willadsen, 2004, de la Fuente and Kocan, 2006, Willadsen, 2008, Parizi et al., 2012).

From a scientific point of view, one of the major limitations in anti-tick vaccine development is the identification of suitable antigen targets. With advances in characterization of tick genomes, along with the use of bioinformatics, RNA interference (RNAi), mutagenesis, immunomapping, transcriptomics, proteomics, expression library

immunization (ELI) and other technologies allowing for a rapid, systematic and comprehensive approach to tick vaccine discovery, this constraint is being surpassed (de la Fuente and Kocan, 2006, Domingos et al., 2013). Although the availability of these powerful techniques the identification and characterization of effective antigens remains a significant challenge.

1.5 Tick-borne diseases

In history, Theobald Smith and Frederick L. Kilbourne (1889 to 1893) demonstrated how a disease was spread from cattle to cattle by ticks serving as the vector of transmission. Moreover, they were able to identify the pathogen of Texas fever, an intra-erythrocytic protozoan which Smith named *Pyrosoma bigeminum* now renamed genus *Babesia* Babes, 1888 (Assadian and Stanek, 2002). Some years later it was established that ticks are able to transmit disease to humans. First reports are even prior to the discovery of Smith and Kilborne and associate the bite of soft ticks in Angola and Mozambique to disease (Livingstone, 1857, Schwan et al., 2012) but only in the beginning of the twenty century route of transmission of the ethological agent of Rocky Mountain Fever was proven to be a tick, the hard tick *Dermacentor andersoni* Stiles, 1908 (Ricketts, 1991, Amsden et al., 2005). Throughout the last century many different associations tick-pathogens were discovered, including bacteria, viruses, protozoan parasites (Jongejan and Uilenberg, 2004, de la Fuente et al., 2008a), nematodes (de la Fuente et al., 2008a, Brianti et al., 2012) and one *Trypanosoma* species (Morzaria et al., 1986, Latif et al., 2004); the importance of these arthropods by themselves and as vectors is now very well documented and extensively reviewed (Estrada-Pena and Jongejan, 1999, Jongejan and Uilenberg, 2004, de la Fuente et al., 2008a, Colwell et al., 2011, Dantas-Torres et al., 2012). Table 2 shows ticks identified in Portugal mainland and their associated hosts and pathogens.

Table 2: Ticks identified in Portugal mainland: associated hosts and vector role.

Ticks in Portugal	Most Common associated Host	Most commom associated pathogens	Ref
Argas vespertilionis (Latreille, 1802)	Bats	Issyk-Kul Fever virus; borreliosis	1,2,3
Ornithodoros maritimus (Vermeil and Marguet, 1967)	Birds	Soldado virus	1,2,3
Ornithodoros erraticus (Lucas 1849)	Pigs; Humans	African swine fever virus; Qalyub virus; *Borrelia microti, Borrelia hispanica, Borrelia crocidurae; Babesia meri*	1,2,3,5
Ixodes acuminatus (Neumann 1901)	Wild carnivores and rodents (occasionally birds)		1,2,6
Ixodes arboricola (Schulze & Schlottke, 1930)	Birds	*Rickettsia spp.*	6
Ixodes bivari (Santos Dias 1990)	Rabbit		1,2
Ixodes canisuga (Johnston, 1849)	Dogs, cats, wild carnivores		1,2
Ixodes frontalis (Panzer, 1798)	Wild birds	*Candidatus* Neoehrlichia mikurensis	1,2,6,7
Ixodes hexagonus (Leach 1815)	Domestic and wild carnivores; Ungulates	*Anaplasma phagocytophilum; B. burgdorferi s.l.*	1,2,3,5
Ixodes ricinus (Linnaeus,1758)	Domestic and wild mammals; Humans	*Anaplasma phagocytophilum; Borrelia burgdorferi s.l., Rickettsia helvetica; R. monacensis; Babesia microti; B.divergens*	1,2,3,5
Ixodes simplex (Neumann, 1906)	Bats		1,2
Ixodes ventalloi (Gil Collado, 1936)	Domestic and wild mammals (carnivores), birds, rodents	*Anaplasma phagocytophilum; Rickettsia helvetica*	1,2
Ixodes vespertilionis (Koch, 1844)	Bats	*Issyk-Kul Fever virus*	1,2,3
Dermacentor marginatus (Sulzer, 1776)	Domestic and wild mammals (ungulates, carnivores); Humans	*Rickettsia slovaca; Borrelia lusitaniae; Babesia canis; Coxiella burnetti*	1,2,3,5
Dermacentor reticulatus (Fabricius, 1794)	Domestic and wild mammals, ungulates and carnivores	*Babesia canis; B. caballi; Rickettsia slovaca; Francisella tularensis*	1,2,3,5
Haemaphysalis hispanica (Gil Collado, 1938)	Lagomorphs		1,2
Haemaphysalis inermis (Birula, 1895)	Ungulates		1,2
Haemaphysalis punctata (Canestrini & Fanzago, 1878)	Ungulates, birds	*Palma virus; Crimean-Congo Hemorrhagic Fever virus*	1,2,3,8
Rhipicephalus bursa (Canestrini & Fanzago, 1878)	Domestic dog, ungulates; Humans	*Babesia ovis; B. bigemina; Anaplasma marginale; A. ovis; Theileria ovis*	1,2,3,5
Rhipicephalus pusillus (Gil Collado, 1936)	Domestic and wild mammals (carnivores, lagomorphs ungulates)	*Rickettsia sibirica*	1,2
Rhipicephalus sanguineus (Latreille, 1806)	Wild and domestic mammals (carnivores, insectivores, ungulates and rodents)	*Anaplasma platys; Babesia canis; B. vogeli;B. gibsoni; Ehrlichia canis; Rickettsia conorii; R. massiliae;Hepatozoon canis*	1,2,3,5
Rhipicephalus annulatus (Say, 1821)	Ungulates, carnivores, domestic birds, and lagomorphs	*Babesia bigemina; B. bovis; Anaplasma marginale*	1,3,5,9
Hyalomma lusitanicum (Koch 1844)	Domestic and wild mammals (ungulates, insectivores, carnivores)	*B. burgdorferi s.l*	1,2,9
Hyalomma marginatum (Kosh, 1844)	Ungulates; Birds	*Theileria annulata; Rickettsia aeschlimannii; Borrelia lusitaniae;* Dhori viruses; Crimean-Congo Hemorrhagic Fever virus	1,2,3,5

References [1] (Caeiro, 1999); [2] (Santos-Silva et al., 2011); [3] (de la Fuente et al., 2008a); [4] (Lisboa et al., 2009); [5] (Jongejan and Uilenberg, 2004); [6] (Norte et al., 2012); [7] (Movila et al., 2013); [8] (Dilcher et al., 2012); [9] (Estrada-Pena and Santos-Silva, 2005)

1.5.1 Viral diseases

Ticks can transmit arboviruses which are almost exclusively RNA viruses. The only exception is a DNA virus causing African swine fever. Viruses usually persist in the tick body through out their whole life (Davies et al., 1986). Because of the longevity, ticks are excellent reservoirs for the viruses they carry. Infection can be passed to the subsequent life stages (transstadially) or following generations (transovarially). However, high level of vertical transmission has not been recorded for any tick-borne virus (Turell, 1988). Tick borne encephalitis (TBE) is an important disease affecting also humans, that

compromises the central nervous system tissues, transmitted mainly by *I. ricinus* and *I. persulcatus* Schulze, 1930 (Dumpis et al., 1999).

1.5.2 Bacterial diseases

Ticks also transmit several important bacterial diseases. They are traditionally gathered into two groups: tick-borne rickettsioses (*Rickettsia* spp.) and other bacterial diseases (Sonenshine, 1991). From the first group diseases like Mountain Spotted Fever (*Rickettsia rickettsia*), Boutenneuse Fever (*R. conorii*), Anaplasmosis (*Anaplasma* spp. Theiler, 1910), and Ehrlichiosis (*Ehrlichia* spp. Moshkovski, 1945) are good examples. Rickettsia species parasite generally the endothelial cells lining of the vascular system, *Ehrlichia* invades monocytes, *Cowdria* neutrophils, *Anaplasma* erythrocytes. *Anaplasma marginale* is the primary cause of anaplasmosis in livestock. It is considered to be one of the most important diseases of cattle and sheep (Kocan et al., 2010). During feeding, the gut infections remain constant (Kocan et al., 1992) and the gut cells serve as a reservoir for repeated infection of the salivary glands. This pattern is significantly different from piroplasms (addressed in the next section) where the gut cells are cleared of the infection as the parasites move to the salivary glands. Infection could be transmitted transstadially, but the transovarial transmission of anaplasmosis does not appear to occur (Kocan et al., 1985). Other tick-borne bacterial diseases include for example Lyme disease (caused by the coil-shaped Gram-negative spirochete *Borrelia* Swellengrebel, 1907: *B. burgdorferi s.s., B. afzelii,* and *B. garinii* among others), and Tularemia (caused by the gram-negative coccobacillus *Francisella tularensis*). Lyme disease (borreliosis) is an emerging disease and the most frequent and important human tick-borne disease (Schuijt et al., 2011, Embers and Narasimhan, 2013). These bacteria are transmitted by ticks from the genus *Ixodes*: *I. ricinus* Linnaeus, 1758 or *I. persulcatus* in Europe and *I. scapularis* Say, 1821 or *I. pacificus* Cooley & Kohls, 1943 in North America. A curious fact regarding this disease is that a recombinant vaccine against a *Borrelia* sp. outer surface protein A, OspA, has been developed in the USA in 1998 (GlaxoSmithKline), but was withdrawn from the market essentially due to the lack of demand. In Europe, variability of the OspA among different pathogenic species is often used as an argument against the development of an OspA vaccine (Schuijt et al., 2011, Embers and Narasimhan, 2013). Recently, a tick midgut protein called TROSPA has been shown to be involved in the binding of the

Borrelia surface protein OspA (Pal et al., 2000, Pal et al., 2004). *Borrelia* spp. spirochetes at first multiply within the midgut fluid after ingestion with the blood meal, then stop and remain attached to the digestive epithelium as the infected tick digests the blood meal and molts to the next stage (transstadial transmission). During the next tick feeding, the spirochetes are activated, multiply and penetrate into the hemolymph (expression of OspC instead of OspA (Schwan and Piesman, 2002). Within 48 hours after the attachment, the spirochetes can be found adjacent to the salivary glands (Zhu, 1998).

1.5.3 Protozoan diseases

Tick-borne protozoan parasites of the phylum *Apicomplexa* are important parasites causing disasterous effects and substantial financial losses regarding livestock. In addition, these organisms could infect humans (*Babesia* spp. transmitted by *Ixodes* spp. ticks) (Skrabalo and Deanovic, 1957, Garnham et al., 1969), pets and other animals (*Hepatozoon* spp. Miller, 1908) (Patton, 1908, Jongejan and Uilenberg, 2004). Apicomplexan parasites of economic importance that are transmitted by ticks comprise *Babesia* and *Theileria* spp. Due to the existence of pear-shaped intraerythrocytic stages; they have been referred to as piroplasms (Florin-Christensen and Schnittger, 2009, Schnittger et al., 2012). A unique morphological characteristic shared by piroplasmids with other apicomplexan protozoans is the presence of an apical complex: a complex cellular apparatus consisting of conoid, rhoptries, micronemes (*Babesia*) or microspheres (*Theileria*), and other subcellular organelles (Kakoma and Mehlhorn, 1994). These two piroplasms are phylogenetic close (Allsopp et al., 1994, Florin-Christensen and Schnittger, 2009, Schnittger et al., 2012) and sometimes confusion arises when classifying these genera (Lack et al., 2012). Regardless of this discussion, *Babesia* spp. are typically differentiated from *Theileria* spp. based on life-cycle characteristics, including distinctions in their biology within tick vectors, the manner by which they are transmitted from vector to vertebrate host, and the location of replication in the vertebrate hosts (*e.g.*, *Babesia* spp. multiply only in erythrocytes, while *Theileria* spp. enter lymphocytes and develop into schizonts) (Uilenberg, 2006).

The notable impact of *Babesia* spp. infections in three host groups: domestic animals, humans and, most recently acknowledged, some wildlife species make of this

pathogen one of the most important protozoan transmitted by ticks (Schnittger et al., 2012).

1.6 The Genus *Babesia*

It was at the end of the 19th century that the Romanian biologist Victor Babeş discovered micro-organisms in erythrocytes of cattle in Romania and associated them with bovine hemoglobinuria or red water fever (Babes, 1888). Five years later *B. bigemina* was recognized as the causative agent of Texas Cattle Fever (Smith and Kilborne, 1893).

Babesiosis is caused by intraerythrocytic parasites of the Apicomplexan genus *Babesia* and is a common infection of vertebrate animals worldwide. Currently it is known that members of the genus *Babesia* are one of the most ubiquitous and widespread blood parasites in the world, second only to the trypanosomes. All over the world, there are more than 100 *Babesia* species. (Homer et al., 2000, Hunfeld et al., 2008, Gohil et al., 2013). Ticks from several genera are now known to be vectors and reservoirs of numerous *Babesia* spp. transmissible to reptiles, birds and mammals (Gohil et al., 2013).

The *Babesia* species are characterized by transovarial transmission in the vector tick and the limitation of infecting only erythrocytes in the host (Uilenberg, 2006). Completion of a life cycle and therefore the maintenance of *Babesia* parasites are completely dependent on both the tick and the vertebrate host (Mehlhorn and Schein, 1984, Chauvin et al., 2009) and therefore, the distribution of all the different *Babesia* species is primarily ruled by the geographical distribution of their tick vectors. *Babesia* organisms can be visualized in a Giemsa stained infected blood smear. They are pyriform (pear shaped) or observed as round or amoeboid forms. Depending on the *Babesia* species, a host erythrocyte may be parasitized by single, paired, or multiple organisms. The size of the organisms varies depending on the species being classified as either small (1.0-2.5 μm; *B. bovis*, *B. gibsoni*, *B. ovis* and *B. divergens*) or large *Babesia* (2.5-5.0 μm; *B. bigemina*, *B. caballi* and *B. canis*), accordingly to the size of the trophozoites (Urquhart et al., 1996, Chauvin et al., 2009). These morphological categorizations are usually consistent with the phylogenetic characterization based on nuclear small subunit-

ribossomal RNA gene (18S rDNA) sequences, showing that small and large *Babesia* parasites are divided in two different phylogenetic clusters (Homer et al., 2000).

1.6.1 Bovine babesiosis

The major economic impact of babesiosis is shown in cattle industry. Bovine babesiosis, in which the most important pathogens are *B. bigemina*, *B. bovis* and *B. divergens* (prevalent only in Europe and potentially zoonotic), threaten the health and safety of millions of bovines in tropical and subtropical regions of the world (Bock et al., 2004). References, to what it may have been a babesial infection, were found in the biblical book of Exodus (Homer et al., 2000).

Since the discovery of this disease a great amount of time and money have been spent to eradicate/control this type of cattle disease since it can induce animal mortality, abortions, reduction of milk/meat production, and sometimes, neurological symptoms (Brown and Palmer, 1999, Suarez and Noh, 2011). If in one hand we have the economic losses related to the disease on the other we will have the high cost of tick control, disease detection, prevention and treatment (de Leon et al., 2010, Mosqueda et al., 2012). Furthermore there is an indirect and underestimated cost of the disease related with the refusal of cattle farmers in endemic areas to improve the production of beef and milk in their herds by introducing pure-breed animals, most of them from tick-free areas, because they will present an acute form of the disease and many will die in the following weeks to their arrival. The consequence is that the quality of cattle in endemic areas remains low, therefore impeding the development of the cattle industry and the well being of producers and their families (Mosqueda et al., 2012).

In almost a century and a quarter since the first report of the disease there is none safe and efficient vaccine available, chemotherapeutic choices are limited and there are few low-cost, reliable and fast detection methods (Mosqueda et al., 2012).

Nowadays, in Portugal mainland there are an estimated total of ~3.9 million food animals with economic importance. From them ~2.8 million are ovine and caprine, and

1.1 million are bovine, which are susceptible to tick infestation and consequently, to infection with tick-borne diseases including babesiosis (Silva et al., 2010a). From the ticks found to parasitise Portuguese cattle three, *Rhipicephalus (Boophilus) annulatus, R. bursa* Canestrini & Fanzago, 1878 and *Ixodes ricinus* are known vectors for babesiosis (Estrada-Pena and Santos-Silva, 2005). Three fairly different studies confirm the presence of bovine babesiosis in Portugal. First a quite small study in 100 bovines report the presence of about 76% of *Babesia* spp. in blood smears with reference to mixed infections with *Anaplasma* spp.(29%) and *Theileria* spp.(5%) (Antunes, 2008). The second and more relevant study comprising a molecular methodology shows that in 1104 cattle blood samples collected from Central and Southern regions of Portugal and analyzed by PCR–reverse line blotting (RLB), 5.2% were positive for *Babesia* spp. Furthermore *B. divergens* was the most prevalent with 4.2% and several mixed infections were detected (Silva et al., 2010a). In 2013 a methodologically similar study in approximately 1400 bovines confirmed the presence of babesial infections, with 7, 9% of the studied population but remarkably most of the infections (7,8%) are *B. bigemina* (Gomes et al., 2013). Despite some differences these studies demonstrate the presence and dispersal of bovine babesiosis in Portugal.

1.6.2 Symptoms of bovine babesiosis

Despite being closely related, *B. bovis* and *B. bigemina* cause remarkably different symptons in cattle. The clinical signs associated with *B. bovis* infections are fever, inappetence, depression, increased respiratory rate, weakness and a reluctance to move. Haematuria is often present (red water). Anaemia and jaundice develop especially in more protracted cases. Cerebral babesiosis is characterized by convulsions, hyperaesthesia and paralysis, due to sequestrations of parasites in the brains capillaries, resulting in low parasitaemia level (less than 1%) in circulating blood and is manifested by a variety of signs of central nervous system involvement and the outcome is almost invariably fatal (Bock et al., 2004). In *B. bigemina* infections, pathogenesis is almost entirely related to rapid, and sometimes massive, intravascular haemolysis. Infection usually leads to a less pathogenic disease, even though parasitaemia often exceeds 10%. Haemoglobinuria is characterisitic of this infection. There is no cerebral involvement and recovery in non-fatal cases is usually rapid and complete. However, in some cases the

disease can develop very rapidly with sudden and severe anaemia, jaundice and death (Bock et al., 2004).

1.6.3 Diagnosis of bovine babesiosis

Diagnosis of clinical cases of babesiosis can be achieved by microscopy, immunological or using molecular detection methods (Mosqueda et al., 2012). From the first group blood smears stained with Giemsa or acridine orange can be highlighted. Thin blood films from *B. bovis* are prepared from capillary blood, as blood of general circulation may contain up to 20 times fewer parasites due to sequestration of infected erythrocytes in capillaries of brain and other organs (Bose et al., 1995). In *B. bigemina* infections, parasitized cells are evenly distributed throughout blood circulation. For low levels of parasitemia, especially in cases where *B. bovis* is involved, diagnosis is based on thick smears of infected blood stained with Giemsa (Morzaria et al., 1992, Mosqueda et al., 2012). The advantage of the thick smear is that a large amount of erythrocytes is analyzed in a reduced amount of space; therefore the probability of finding infected cells is ten times higher than in a thin smear. These techniques are inexpensive and reasonably portable although accuracy of diagnosis relies on the training and skills of the microscopist.

Different immunological tests have been described for *Babesia* spp. detection, as the indirect immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) and the immunochromatographic test (ICT). All are based on the recognition of parasite antigens by serum antibodies in the blood of the tested animal. ELISA methods include the use of recombinant antigens and monoclonal antibodies, thus increasing specificity and diminishing unspecific binding and signal (Goff et al., 2008, Mosqueda et al., 2012). The ICT is a rapid diagnostic device that detects antibodies against a specific antigen in a small amount of serum by means of specific antibody and a recombinant antigen both impregnated on a nitrocellulose membrane-based test strip (Weigl et al., 2008). This test shows promise since it is very easy to perform and read, does not require a trained technician since it does not use any special equipment, can be implemented in the field and has a low cost comparable with

other techniques (Mosqueda et al., 2012). Immunological methods to detect *Babesia* spp. parasites have the disadvantage of relying on the presence of specific antibodies against those parasites, which may take days or weeks to develop in an infected animal or they are present for months after the infection has disappeared, making their usefulness very limited in acute disease cases, vaccinated or cleared-by-treatment animals.

The different molecular diagnosis methods rely on the presence and amplification of pathogen DNA (PCR based assays) so they can distinguish active infections. With the evolution of more sensitive PCR based techniques, several methods for the detection and differentiation of bovine babesiosis infections have been described, including nested PCR (Figueroa et al., 1993), reverse line blot (RLB) hybridization (Gubbels et al., 1999), LAMP (Loop-Mediated Isothermal PCR) (Iseki et al., 2007) and real time PCR (Buling et al., 2007, Criado-Fornelio et al., 2009). Despite the advantages of these techniques regarding sensitivity, due to one factor or another (costs, contaminations, validation), none of these methods is used globally.

1.6.5 *Babesia bigemina* life cycle

The complex life cycle of *Babesia* spp. takes place in two hosts, vector and mammalian host, and sexual and asexual reproduction proceeds through three stages, (i) gamogony – sexual development with formation and fusion of gametes inside the tick gut, (ii) sporogony - asexual reproduction in tick salivary glands, (iii) merogony - asexual reproduction in the vertebrate host. *B. bigemina* life cycle (similar to the life cycle of *B. bovis*) is described in figure 7.

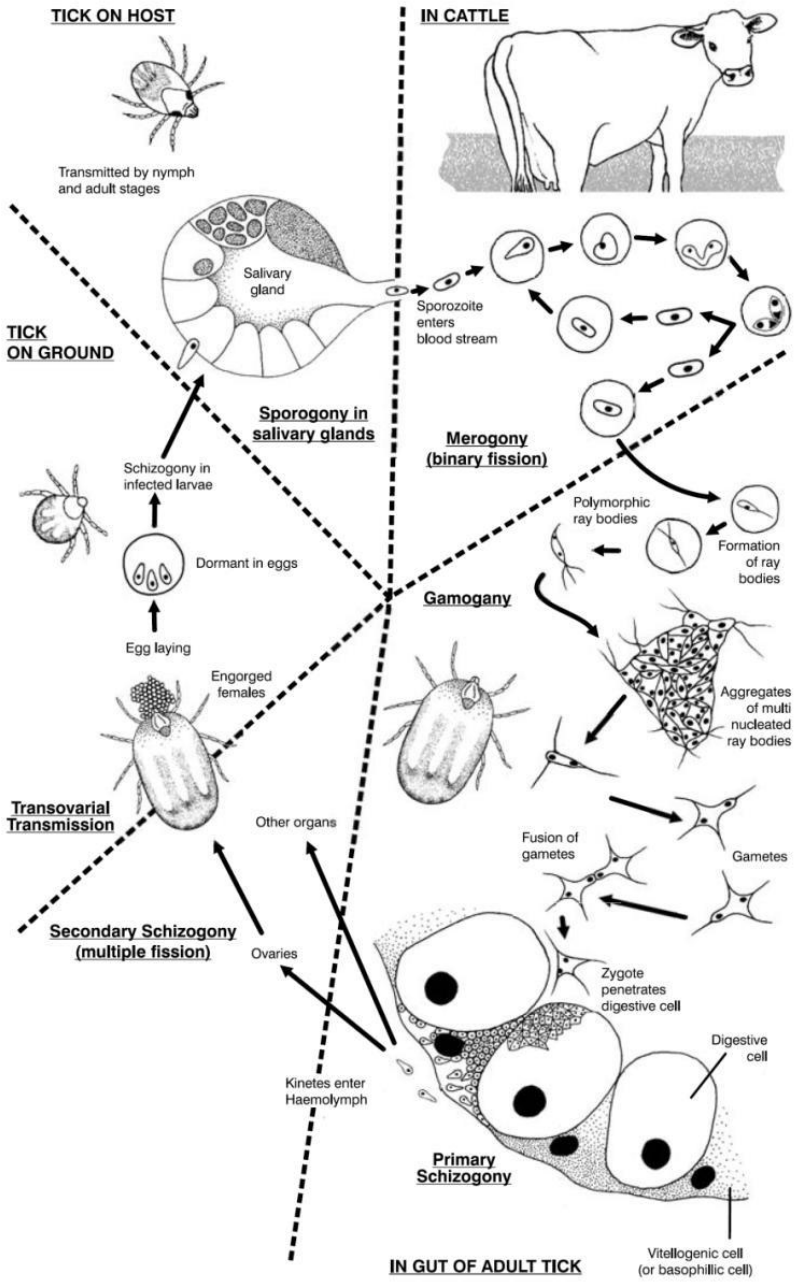

Figure 7: The life cycle of *Babesia bigemina* in cattle and the ixodid tick vector *Rhipicephalus (Boophilus) microplus*.
(adapted from Bock et al, 2004).

1.6.5.1 Events in the vertebrate host

Both *B. bovis* and *B. bigemina* sporozoites enter the host with the saliva of the infected tick larvae (*B. bovis*), nymph or adult (*B. bigemina*) (Callow and Hoyte, 1961a, Suarez and Noh, 2011). Each sporozoite penetrates the cell membrane of an erythrocyte with the aid of a specialized apical complex, forming a parasitophorous vacuole (Sibley,

2004). The vacuole membrane gradually disintegrates, and the parasite is left with the defining piroplasm feature of a single membrane, in contrast to *Plasmodium* species, which invade by a similar mechanism but retain the host membrane in addition to its own (Rudzinska et al., 1976, Homer et al., 2000). Once inside the erythrocyte, it transforms into a trophozoite, by binary fision, from which two merozoites develop by a process of merogony. The merozoites lyse the cell and go on to infect additional erythrocytes. Four parasites can be formed at the same time giving rise to a Maltese cross form which is characteristic in *Babesia* sp. infection. Rapid reproduction destroys the host cell and leads to hemoglobinuria in the host. Multiplication is asynchronous and different divisional stages can be seen in the bloodstream at the same time (Chauvin et al., 2009). Some trophozoites develop into a diploid ovoid type of merozoite, called a gamont precursor. These gamont precursors do not develop further until they are taken up by the tick in the blood meal (Mackenstedt et al., 1995, Chauvin et al., 2009) later on, when in the gut of the tick, even prior to leaving the erythrocytes these precursors develop into gametocytes (Homer et al., 2000).

1.6.5.2 Events in the tick

When *Babesia*-infected erythrocytes are ingested by ticks, most of the parasites degenerate and are destroyed. However, environmental changes in the passage from host blood to the midgut of the tick vector stimulate the development of "pre-gametocytes" into elongated bodies, with arrowhead-shaped ray appearence, believed to be gamonts, the so-called "ray bodies" ("strahlenkörper") (Chauvin et al., 2009). The ray bodies undergo further multiplication within the consumed erythrocyte and form large aggregations of multinucleated ray bodies. After the consumed erythrocyte is digested, and once gametogenesis is complete, single-nucleated and haploid gametes emerge from the aggregates (Mackenstedt et al., 1995). The gametes fuse in the lumen of the tick's digestive tract to form an elongated zygote. Once the zygote reaches the cell membrane invaginates at the point of contact. No parasitophorous membrane is produced and the midgut cell membrane appears to be disrupted at the point of entry, apparently due to the action of enzymes released from a coiled structure of the invading parasite (Chauvin et al., 2009). At some timepoint, the zygote undergoes one-step meiosis to form a haploid

zygote (Mackenstedt et al., 1995) and motile kinetes are formed (primary schizogony). These organisms escape the midgut epithelium into the haemolymph and infect a variety of cell types and tissues, including the ovaries where successive cycles of secondary schizogony take place (Mackenstedt et al., 1995, Homer et al., 2000). Consequently, transovarial transmission occurs with further development taking place in the larval stage. Sporogony takes place at each tick stage and the *Babesia* sp. infection acquired during one life stage is passed on to the next (transtadial transmission). In the salivary glands kinetes are transformed into multinucleated stages and break up to form sporozoites (Mackenstedt et al., 1995). In all species, sporozoite development usually begins when the infected tick attaches to the vertebrate host. In *B. bigemina*, infective sporozoites take about 9 days to appear and therefore transmission only occur in the nymphal and adult stages of the tick (Callow and Hoyte, 1961b, Hoyte, 1961, Suarez and Noh, 2011). In the case of *B. bovis*, the formation of infective sporozoites usually occurs within 2 to 3 days of larval tick attachment (Suarez and Noh, 2011) and transmission occurs only in this stage as infective sporozoites do not persist beyond the larval stage (Mahoney and Mirre, 1979, Bock et al., 2004).

1.6.6. Hosts immunity to *Babesia* sp.

1.6.6.1 In the vertebrate host

The mechanisms of immunity to *Babesia* spp. require both innate and adaptive responses (Brown et al., 2006b, Shkap et al., 2007a). The innate immune response involves activation of macrophages, suppressing proliferation of infected cells, production of the tumor necrosis factor a (TNF-a), nitric oxide (NO) and natural killer cells (cytotoxic lymphocyte). Calves are more resistant to disease than older cattle, and this age-linked resistance is thought to be the result of natural killer cell responses in the spleens of young animals (Brown et al., 2006a, Shkap et al., 2007a). The mechanisms of acquired immunity seems to rely on the rapid activation of memory and effector $CD4^+$ T helper cells that secrete IFN-ɣ (interferon gamma), thereby activating phagocytic cells and enhancing antibody production by B cells (Norimine et al., 2003).

In the vertebrate host two different types of infection can be established: (a) primary infection, which is conditioned by the host genetic resistance to the pathogen (species breed, vertebrate host status, age) and (b) re- infection, process associated with the resistance to the re-infection acquired as a result of the continuous presence of the parasite which can persist for several months or even years at a very low level of parasitaemia maintaining the immunological response active (Chauvin et al., 2009).

The clinical expression of babesiosis is highly linked to the status of the individual animal. Different breeds of cattle are known to differ in susceptibility to infection and manifestation of clinical signs of babesiosis. *Bos taurus* breeds are more susceptible to both *B. bigemina* and *B. bovis* than *Bos indicus* (Bock et al., 1997). Calves exposed to babesiosis during the first 6 to 9 months rarely show clinical symptoms and develop a solid long-lasting immunity (de Waal and Combrink, 2006). The resistance of young animals to clinical signs is seen as an important factor for the maintenance of enzootic stability. Compared to most other infectious diseases, which affect juveniles more severely than adult animals, inverse age resistance is unusual and the underlying mechanisms of this phenomenon are still poorly understood (Zintl et al., 2005).

Clearly, for a vector-borne parasite, establishing persistent reservoir infections early in the life of the host is a good strategy favoring further transmission. Acute development or parasite clearance from the blood stream could be interpreted as a mal adaptation in the *Babesia* vertebrate host interface. A delayed, unsuitable or insufficient immune response could lead to clinical manifestations of babesiosis and eventually to death of the infected host, making pathogen transmission impossible. Transmission to ticks at a low level of parasitemia must be the prevalent modality of *Babesia* spp. transmission in the field, given the long-term maintenance of the parasite in a biotope and generally asymptomatic carriage. The establishment of low and long-lasting parasitemia increases the duration of the transmission period and thus the chance of being acquired by the tick (Chauvin et al., 2009).

1.6.6.2 In the tick

Invertebrate animals lack an adaptive immune system and their defense against potential pathogens has to rely on a network of cellular immune reactions (*e.g.*, phagocytosis, encapsulation and nodulation) and humoral factors involved in pathogen recognition and elimination (pattern recognition receptors, lectins, complement-like system, pro-phenoloxidase activation, hemolymph coagulation, antimicrobial peptides, reactive oxygen species among others (Kopacek et al., 2010). Knowledge of innate immunity in ticks is still insufficient (Kopacek et al., 2012).

As referred before *Babesia* spp. present transstadial transmission which can be considered as a specific adaptation to the tick life cycle, which involves only one blood meal per tick stage (*i.e.* larva, nymph and adult in *Ixodes ricinus* a three host tick), allowing parasite transmission during each of those stages; transovarial transmission is more rarely observed in tick-transmitted pathogens and is a characteristic of the *Babesia* genus as compared with *Theileria* spp.. Transovarial transmission is considered an adaptation for long-lasting persistence, because some ticks remain infected and infective for several generations without needing to again feed on infected animals (increasing transmission efficiency) (Chauvin et al., 2009).

High levels of infection of ticks with *Babesia* parasites can have strong detrimental effects on these vectors (Hoffmann, 1971, Gray, 1982, Cen-Aguilar et al., 1998 Chauvin et al., 2009, Florin-Christensen and Schnittger, 2009), however it seems that this long lasting co-evolution of ticks and pathogens resulted in a mutual tolerance, apparently adapted to tick physiological differences (Mans, 2011, Hajdusek et al., 2013).

Successive stages of the life cycle of *Babesia* sp. parasites take place in different compartments of *Ixodidae* ticks. For this to occur, these parasites need to cross multiple barriers such as midgut and salivary gland epithelium, and, due to their transovarian mode of transmission, also the ovary epithelium (Florin-Christensen and Schnittger, 2009).

1.6.4 Babesiosis control: Treatment and vaccination

Babesiosis control is essential, due to its huge implications in livestock production and in public health issues (Bock et al., 2004). Nowadays, with the introduction of exotic breeds, babesiosis control is even more important, because those breeds usually do not show natural immunity against *Babesia* spp (Graf et al., 2004). Control of bovine babesiosis can be achieved either by tick management, immunization, anti-babesia drugs administration or by a combination of these approaches (Suarez and Noh, 2011).

1.6.4.1 Chemotherapy

Chemotherapy is generally effective against bovine babesiosis, a large number of chemical compounds have been reported to be active against bovine *Babesia* parasites (Vial and Gorenflot, 2006). Successful treatment depends on early diagnosis and the prompt administration of drugs. Current treatments provide protection from disease but usually allow a sufficient level of infection (low level parasitaemias) for immunity to develop which is important in areas where babesiosis is endemic. Only a few babesiacides are now available commercially and diminazene aceturate and imidocarb dipropionate are the most widely used:

Diminazene aceturate– used intramuscularly at a dose of 3.5 mg/kg for treatment (de Vos, 1979). This drug is the most used anti-trypanosomal agent (Peregrine, 1994) and it has also been used in the treatment of bovine babesiosis. Diminazene binds irreversibly to double-stranded DNA. The type of binding between diminazene and DNA is non-intercalative and has high affinity for kinetoplastid DNA (kDNA), which impairs kinetoplast replication and function (Brack et al., 1972, Mosqueda et al., 2012).

Imidocarb - used subcutaneously at a dose of 1.2 mg/kg for treatment; it is also used as chemoprophylactic, at a dose of 3 mg/kg, which will prevent clinical infection from *B. bovis* for 4 weeks and *B. bigemina* for at least 2 months (Taylor and McHardy, 1979). The mode of action of imidocarb is not fully understood, however, two mechanisms have been proposed: interference with the production or use of polyamines (Bacchi et al., 1981), or the prevention of entry of inositol into the parasitized erythrocyte, producing starvation of the protozoan (McHardy et al., 1986). This drug is associated with residue

problems; several studies have showed that imidocarb is retained in edible tissues of ruminants for long periods after treatment (McHardy et al., 1986, Coldham et al., 1994, Suarez and Noh, 2011, Mosqueda et al., 2012). At the high dose of 3 mg/kg, imidocarb completely eliminate *B. bovis* and *B. bigemina* leaving the animals susceptible to reinfection and for this reason reduced drug levels are sometimes indicated (Bock et al., 2004, Vial and Gorenflot, 2006), especially in endemic areas where the development of protective immunity is desirable. But, the use of reduced drug levels increases the risk of development of resistance against the drug when used extensively (Rodriguez and Trees, 1996).

Recently, several pharmacological compounds were developed and evaluated, offering new options to control the disease like triclosan (chlorinate aromatic compound) (Bork et al., 2003), nerolidol, also known as peruviol, a sesquiterpene present in essential oils of several plants (AbouLaila et al., 2010), artemisinin and its derivatives (Goo et al., 2010). Trifluralin derivatives specifically bind alpha-tubulin in plants and protozoa parasites causing growth inhibition and also have been tested in *B. bovis* parasites (Silva et al., 2013). Other new drug targets have been described including cysteine proteases (Martins et al., 2010, Martins et al., 2011) which can inhibit parasite growth suggesting that these targets cannot be overlooked.

1.6.4.2 Vaccines

There are different types of vaccines against *Babesia* sp. including attenuated live (calf-derived and culture-derived) and recombinant vaccines. Live attenuated vaccines for cattle are used with over 95% efficacy from a single vaccination (Bock et al., 2004). Attenuated vaccines can be produced by parasites multiple passages *in vivo* in splenectomized calves, the calf-derived vaccines (Bock et al., 2004, de Waal and Combrink, 2006) or by parasites growth *in vitro*, the culture-derived vaccines (Jorgensen et al., 1989, Shkap et al., 2007a). Calf-derived vaccines have several associated concerns, such as the possible spread of silent pathogens, difficulties in standardizing vaccine dose, risk of virulence reversion, maintenance of carrier animals, quality of vaccine production, maintenance, transportation (Shkap et al., 2007a) and short shelf-life (Bock et al., 2004), limitations of use in animals older than 8-9 months, adverse effects (de Waal and

Combrink, 2006) and potential risk of parasite transmission, since vaccinated cattle remain persistently infected for several months (Pipano, 1995). Culture-derived vaccines do not have the risk of pathogens spreading culture, and their main disadvantages remain in the need to fresh bovine erythrocytes and serum from specific donors, which have to be maintained in highly strict conditions and in the fact that vaccines can lose their immunogenicity and virulence in a long-term cultivation (de Vos, 1979). There are several studies reporting the use of these attenuated vaccines (Shkap et al., 2007b, Fish et al., 2008, Ojeda et al., 2010) and despite some disadvantages, live attenuated strains of *B. bovis* and *B. bigemina* have been used for many years, because they offer a long-lasting protection (Benavides and Sacco, 2007). The lack of understanding of immune mechanisms to primary and secondary infection and the fact that many protozoa have developed elaborate mechanisms such as antigen variation for evading host immunity remain obstacles to developing effective vaccines using recombinant technology (Jenkins, 2001). Antibodies against a recombinant microneme protein from *B. bovis* (Bbo- MIC1) secreted on parasite surface, inhibit erythrocyte invasion in *in vitro* cultures also this protein was recognized by antibodies in serum of *B. bovis* infected cattle, showing the immunogenicity of Bbo-MIC1 and its use as potential vaccine (Silva et al., 2010b). Antibodies directed to an apical membrane antigen 1 (AMA-1), reduced the invasion efficiency *in vitro*; moreover, this molecule is highly similar to another AMA-1 from *B. bigemina*, indicating that this vaccine could, possibly, have a cross reactivity with other *Babesia* species (Torina et al., 2010).

1.7 Tick and tick borne diseases control

The ultimate goal of arthropod vector vaccines is the control of vector infestations and vector-borne diseases. The effect of vector vaccines on diseases transmitted by vectors could be obtained by (a) reducing vector populations and thus the exposure of susceptible hosts to vector-borne pathogens, (b) reducing the arthropod vector capacity for pathogen transmission, and, preferably, (c) a combination of these factors (Merino et al., 2013). Anti-tick vaccines, affecting vector numbers, could directly influence disease incidence and secondly, since it is increasingly clear that disease transmission can involve complex interactions between host, vector and disease organism, it is possible that by

disturbing the tick the vaccine also will affect the disease (Willadsen, 2004). Identification of antigens essential for both tick survival and pathogen infection and transmission will likely contribute to the discovery of novel vaccine strategies for the simultaneous control of ticks and tick-borne pathogens.

1.7.1 Tick antigens

Tick antigens are usually regarded as either "exposed" or "concealed" antigens. "Exposed" antigens are those that naturally come into contact with the host immune system during tick infestation. Hosts immunized with these antigens are boosted by continuous tick exposure. "Concealed" antigens are not exposed to the host immune system and therefore repeated immunizations are required to maintain elevated antibody titers. However, the advantage of using concealed antigens results from the unlikely possibility that ticks would have evolved a mechanism to effectively counteract the effect of the host immune system as had occurred with exposed antigens (Willadsen and Kemp, 1988, Willadsen, 2004, de la Fuente et al., 2006c, Kiss et al., 2012). An antigen named **64P,** identified in *R. appendiculatus* Neumann 1901, seems to explore the advantages of both antigens types. 64P is a 15 kDa protein that resembles mammalian host skin proteins, identified in expression libraries as a putative tick cement protein involved in the attachment and feeding (Trimnell et al., 2002, Havlikova et al., 2009). Vaccination of tick-naıve hosts with the recombinant protein 64P provided significant protection against nymphal and adult infestations, producing 48% nymphal and up to 70% adult mortality, with some effects on engorgement weight and egg masses as well (Trimnell et al., 2005). In hamster, guinea pig, and rabbit models, this cement antigen acts as a dual-action vaccine by targeting the tick-feeding site (impairing attachment and feeding) and cross-reacting with "concealed" midgut antigens, resulting in death of engorged ticks (Labuda et al., 2006, Merino et al., 2013). Moreover, antigenic cross-reactivity of 64TRP from *R. appendiculatus* with its homologues in *R. sanguineus* Latreille, 1806, *I. ricinus*, *Amblyomma variegatum* Fabricius, 1794, and *R. microplus* was confirmed by Western blotting (Trimnell et al., 2005) and was reported that vaccination of mice with 64TRP antigen prevented transmission of tick-borne encephalitis virus by *I. ricinus* consequently having a protective effect on pathogen transmission (Labuda et al., 2006).

Other antigens have been identified and proposed for anti-tick vaccine development. Some examples are herein described. Tick **Subolesin** (SUB) was first discovered in *I. scapularis* (Almazan et al., 2003b) and has a role in tick immunity (Zivkovic et al., 2010b, de la Fuente et al., 2011) and in other molecular pathways (de la Fuente et al., 2008b). This protein is the ortholog of insect and vertebrate akirins (AKR) (de la Fuente et al., 2006a, Goto et al., 2008, Galindo et al., 2009) and has been shown to protect against tick infestations and infection by vector-borne pathogen such as *A. phagocytophilum*, *A. marginale*, *B. bigemina*, and *B. burgdorferi* (de la Fuente et al., 2006b, Merino et al., 2011b, Bensaci et al., 2012). **Ferritins** are iron-storage proteins that play a pivotal role in the homeostasis of iron during tick feeding. The gut-expressed iron storage protein, ferritin 2, without functional orthologs in vertebrates, has been recently characterized as a gut-specific protein secreted into the tick hemolymph, where it acts as an iron transporter (Kopacek et al., 2003). Silencing of ferritin 2 by RNAi showed significant impacts on tick feeding, oviposition and larval hatch (Hajdusek et al., 2010, Hajdusek et al., 2009). Immunization of cattle with a cocktail vaccine containing recombinant *R. appendiculatus* **serpins** RAS-3, RAS-4, and a 36 kDa immune-dominant protein RIM36, reduces tick infestations and also had an effect on the tick mortality rate of *Theileria parva*-infected ticks which increased from 10.8 to 48.5% in the vaccinated group (Imamura et al., 2008). Serpins are known to regulate many important functions such as blood coagulation, food digestion, inflammatory and immune responses (Muleng et al., 2001) and therefore are attractive target antigens for tick vaccine development. C**alreticulin (CRT)**, a calcium-binding protein identified in several species, with exception of yeasts and prokaryotes, whose genomes were totally sequenced (Persson et al., 2002), highly conserved with a 96% amino acid identity for CRT from human, rabbit, rat and mouse (Waser et al., 1997). CRT is known to perform several functions in mammals (Michalak et al., 1999, Michalak et al., 2009) and, in ticks, this protein was identified in salivary glands and tick saliva (Jaworski et al., 1995, Kaewhom et al., 2008). Probably, this protein is essential to ticks feeding and pathogen transmission, through its anti-thrombotic and complement inhibition functions (Kaewhom et al., 2008). All these facts support the possibility of using CRT as an antigen in a vaccine against cattle ticks. The *crt* gene has been characterized, cloned and expressed in different ticks (Xu et al., 2004, Parizi et al., 2009) like *A. americanum* Linnaeus, 1754, *D. variabilis* (Jaworski et

al., 1995) and *R. microplus* (Ferreira et al., 2002). Rabbit's immunization with the recombinant CRT developed necrotic feeding lesions on *A. americanum* challenge (Jaworski et al., 1995) but it was found to be poorly immunogenic in cattle (Ferreira et al., 2002) placing this protein aside an ideal vaccine antigen. Nevertheless, this antigen highly involved in tick´s feeding process as well as pathogen transmission should be thoroughly explored (Parizi et al., 2012).

Although previous studies have identified tick genes related to *Babesia* sp. infection (Rachinsky et al., 2007, Rachinsky et al., 2008, Heekin et al., 2012, Heekin et al., 2013), only few tick genes have been shown to be directly implicated in the vector-pathogen interaction (Hajdusek et al., 2013). Two molecules, involved in tick defense against babesia parasites have been described in *Haemaphysalis longicornis* Neumann, 1901: **longicine**, an antimicrobial peptide produced in the tick midgut epithelium (Tsuji et al., 2008) and **longipain** a cathepsin B protease apparently secreted also in the lumen of the midgut epithelium (Uilenberg, 2006). Recombinant proteins of both molecules, inhibited proliferation of *Babesia* sp. merozoites in *in-vitro* cultures and silencing by RNAi resulted in increased number of parasites in the midgut lumen, ovary, and hatched larvae. Furthermore, RNAi silencing of **subolesin** (mentioned above) or vaccination with recombinant subolesin strongly reduced acquisition of *B. bigemina* by *R. microplus* fed on an infected cattle (Merino et al., 2011b). **Vitellogenin,** a storage protein and source of amino acids during embryogenesis was identified as expressed on the oocyte plasma membrane during ovary development. RNA interference studies have demonstrated its critical role in transovarial transmission of *B. gibsoni* (Boldbaatar et al., 2008) revealing its interest as a vaccine antigen.

1.7.2 Antigens identification

Recent molecular techniques are assisting in the identification of potential tick-protective antigens. Development of high throughput DNA sequencing technologies and bioinformatic tools facilitate assignment of provisional function to expressed sequence tags (ESTs). Recently a new approach was extended to tick research. Deep sequencing of transcriptome, also known as RNAseq, provides both the sequence and frequency of RNA molecules that are present at any particular time in a specific cell type, tissue or

organ (Wang et al., 2009). Despite being a very appealing method, since a complete transcriptomic picture of what's happening in the tick in a specific situation can be obtained, to date only one report was published using this high-throughput sequencing method (Schwarz et al., 2013). Data analysis of this type of sequencing is rather laborious and expensive so alternative methods with, not the same but positive outcomes have been successfully used. An example is the use of cDNA expression library immunization (ELI) (Barry et al., 1995) in combination with sequence analysis of ESTs based on rapid screening of the expressed genes without prior knowledge of the antigens encoded by the cDNAs (Almazan et al., 2003a, Ghosh et al., 2007). This method allows for antigen identification without introducing prior criteria to direct the selection of candidate genes and thus may result in the discovery of novel and unexpected antigens. Briefly, sequential simpler pools of cDNA clones from an *I.scapularis* cell line expression library were immunized in mice followed by a controlled tick challenge leading to the identification of protective pools. Individual clones from the pools that induced immunity were sequenced and compared to sequence databases allowing identification with previously reported genes with known function (Almazan et al., 2003b).

Suppression subtractive hybridization (SSH) is a widely used method for separating DNA molecules that distinguish two closely related DNA samples of either cDNA or genomic DNA nature (Diatchenko et al., 1995, Diatchenko et al., 1999). SSH results in differences in relative abundance of transcripts that are highlighted between populations. The technique relies on the removal of double stranded DNA (dsDNA) formed by hybridization between a control and test sample, thus eliminating cDNAs of similar abundance, and retaining differentially expressed, or variable in sequence, transcripts. Rudenko et al. (2005) used this technique to identify *I. ricinus* genes induced by feeding (*e.g.* cysteine protease propeptide, actin) or *Borrelia burgdorferi* infection (*e.g.* glutathione S-transferase, defensin). Other studies succeeded identifying antigens related to tick feeding and even to pathogen infection (Lew-Tabor et al., 2010, Heekin et al., 2012, McNally et al., 2012, Heekin et al., 2013) but the influence of those genes was not demonstrated in tick survival, reproduction or even on infection process. Gene identification associated with functional analyses of genes like RNA interference can validate antigens as potential targets for further investigations (Zivkovic et al., 2010a).

RNAi or post transcriptional gene silencing, is a conserved and natural process that cells use to turn down, or silence, specific genes (Fire et al., 1998, Montgomery et al., 1998). Small interfering RNAs (siRNAs) are the effector molecules of the RNAi pathway that is initiated by double-stranded RNA (dsRNA) and results in a potent sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as the dsRNA trigger (Mello and Conte, 2004, de la Fuente et al., 2007c). Pioneering observations on RNAi were reported in plants (Ecker and Davis, 1986, Ratcliff et al., 1997), but only when Andrew Fire and Craig Mello published their break-through study on the mechanism of RNA interference in *Nature* in 1998 the mystery was properly unraveled. RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines. Over the past decade, research has developed this phenomenon into a valuable tool used to silence target gene expression and perform functional genomic screens (Fjose et al., 2001).

The first report related to the use of RNAi in ticks belongs to Aljamali et al. (2002) and quickly has become largely adopted for gene-silencing. As for other methods of genetic manipulation are rather limited in ticks (de la Fuente and Kocan, 2006, de la Fuente et al., 2007c) RNAi was revealed to be a valuable tool for studying tick gene function, the characterization of the tick-pathogen interface and the screening and characterization of tick protective antigen (de la Fuente and Kocan, 2006). This technique has been used to study the function of tick proteins at the tick–pathogen interface in a number tick species such as in *I. scapularis* which transmits *A. phagocytophilum* and *B. burgdorferi* (Pal et al., 2004, Ramamoorthi et al., 2005, Sukumaran et al., 2006). RNAi was as well used to study the function of tick salivary gland proteins involved in feeding of *A. americanum* (Aljamali et al., 2003, Karim et al., 2004), *Haemaphysalis longicornis* (Miyoshi et al., 2004) and *I. scapularis* (Narasimhan et al., 2004). The inducer of RNAi, dsRNA, is inserted into nymphal or adult ticks which are then allowed to feed normally. In ticks, four different techniques of the dsRNA delivery are known: a) Injection is the most common method of the dsRNA delivery in ticks. Aljamali et al. (2002) was the first report in which dsRNA was injected into the tick females. Since that time, injections by the Hamilton syringe or microcapillary (Narasimhan et al., 2004) have become a routine;

b) Soaking of isolated tissues (Aljamali et al., 2003, Karim et al., 2005) or cells (Blouin et al., 2008, Barry et al., 2013). The latter representing a great potential in the tick cell cultures, which have been proved to actively intake the dsRNA and induce RNAi. Blouin et al. (2008) showed an efficient knock-down of the subolesin expression in the *I. scapularis* IDE8 tick cell line treated with the dsRNA for 24 hours; c) The RNAi-inducing capillary feeding in ticks was demonstrated by Soares et al., (2005) who showed an efficient gene silencing in *I. scapularis* nymphs. The delivered dsRNA was able to silence the studied gene in salivary glands. The authors thus suggest that the dsRNA penetrated the gut and was delivered to salivary glands and other organs by the hemolymph; d) Virus production of dsRNA. Infection of tick cells with the recombinant RNA virus (Semliki Forest virus) containing the tick-borne Hazara virus (HAZV) nucleoprotein gene in sense or antisense orientation, efficiently inhibited HAZV replication (Garcia et al., 2005) providing evidence that dsRNA can be introduced into ticks by recombinant virus and that RNAi might be the basic antiviral response in ticks, similarly as seen in insects (Keene et al., 2004).

The final validation for identified antigens involved in tick-pathogen interface is their use in field vaccination trials but characterization by vaccination is very expensive especially when dealing with *R. microplus* due to their host specificity (Willadsen et al., 1984) and many constrains when attempting to rear it on laboratory animals (Loomis, 1971). Therefore, cattle are most of the times a requirement for the conduction of laboratorial experiments. A low cost alternative for the testing of new molecules is the direct inoculation of drugs or antibodies against a target protein into ticks. Although it doesn´t totally discard the need for cattle it reduces significantly the number of bovines necessary to conduct a trial with different molecules.

Artificial feeding of ticks is a technique widely used for different purposes (Broadwater et al., 2002; Matsuo et al., 2004, Almazan et al., 2005; Gonsioroski et al., 2012), besides mimicking the natural feeding process of ticks it as the advantage that experimental manipulations can be made to the blood meal. Artificial feeding using capillary tubes can be used in vaccine studies, since polyclonal or monoclonal antibodies can be supplemented to the meal (Almazan et al., 2005; Gonsioroski et al., 2012)

allowing the posterior analysis of their effect in biological parameters. This method appears to be a fast, simple and rather inexpensive way to characterize and select antigens to further use in a field vaccination trial.

The combination of strengths of postgenomic databases and RNAi is leading to a better understanding of tick function and the tick-pathogen interface which is greatly needed to formulate improved tick-control methods.

1.8. Premises and aims of this PhD project

The "One Health" approach recognizes the need for veterinarians, human health professionals, and environmental scientists to work together given the dynamic interface among people, animals, and the environment. This approach is increasingly important for zoonotic diseases, such as TBDs, which rely on animals as reservoirs. As ecological niches flux, opportunities arise for vectors to interact with novel species, allowing infectious agents to broaden both geographic and host ranges. Habitat change has been linked to the emergence of novel human and veterinary disease agents, and can dramatically facilitate expansion opportunities by allowing existing vector populations to flourish and by supporting the establishment of new pathogen maintenance systems (Little, 2013). Ticks are of great medical and veterinary importance as they can transmit a wide variety of infectious agents to different vertebrate hosts (de la Fuente et al., 2008a). In particular bovine babesiosis transmitted by cattle ticks continues having a great economic impact in cattle industry due to the lack of effective control methods (both tick control and disease control). With the premise that improved (*e.g.* multi-antigen) vaccine formulations and the discovery of new tick-protective antigens related to infection will improve control of tick and tick-borne diseases as well as increase our understanding on tick-pathogen interface, this PhD project was designed with three main objectives:

a) The identification of genes differentially expressed in *Babesia bigemina* infected cattle ticks;

b) Validate the influence of antigens in pathogen transmission by functional analysis;

c) Characterize the identified antigens regarding potential inclusion on a multi recombinant antigen vaccine;

To address the first objective subtractive suppression hybridization (SSH) methodology was used to obtain a catalogue of tick genes differentially expressed upon babesial infection. Those genes were analyzed and the differential expression was validated in a *B. bigemina* infected *R. annulatus* population. To assess the importance of antigens in the infection process a RNA interference study was designed, where the expression of selected genes was disrupted. Regarding the characterization of antigens proved to be involved in the infection progression, mono and polyclonal antibodies were raised against the selected antigens and used in immunolocalization studies and lastly in tick artificial feeding. These last assays will allow the evaluation of the effect of host antibodies in the tick during feeding and on the transmission of the pathogen representing preliminary data for recombinant vaccine development.

Material and methods

Rhipicephalus microplus and *R. annulatus* ticks used within the present study were reared and obtained from different laboratories according to availability. Adult ticks were fed on calves specifically for this study and all assays with animals were performed in accordance with standards specified in the Guide for Care and Use of Laboratory Animals.

2.1 Identification of differentially expressed genes in *Rhipicephalus annulatus* female ticks

The uninfected and *B. bigemina*-infected *R. annulatus* ticks used for construction of the SSH library were provided by the Kimron Veterinary Institute, Israel. Two 3-4 months old male Friesian calves free of babesiosis were used to obtain *B. bigemina*-infected and uninfected *R. annulatus* female ticks. Prior to tick infestation, calves were tested for antibodies to *Babesia* spp. infection using an indirect fluorescent antibody (IFA) assay (Shkap et al., 2005) and kept under strict tick-free conditions. One calf was intravenously inoculated with cryopreserved $2x10^8$ *B. bigemina* (Moledet strain). Calf clinical responses were monitored by means of daily examinations of body temperature and packed cell volume (PCV), and microscopy of Giemsa-stained blood films. To obtain infected ticks, *R. annulatus* ticks were fed on the infected calf. Engorged adult female ticks were collected from both infected and uninfected calves after feeding and were allowed to digest the blood meal for 4-5 days and subsequently used.

2.1.1 Tick total DNA, RNA and protein extraction

Ticks were rinsed individually twice in distilled water, once in 75% (v/v) ethanol and once more in water. Each tick was dissected and whole internal organs were placed in a 2 ml tube with 1 ml of Tri Reagent T9424 (Sigma-Aldrich, St. Louis, MO, USA). In summary this reagent, a mixture of guanidine thiocyanate and phenol, uses a convenient single-step liquid phase separation results in the simultaneous isolation of RNA, DNA as well as proteins. After adding chloroform or 1-bromo-3-chloropropane and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases. Tick tissues were homogenized using a 21G × 1½" (0.8 × 40mm) needle and 1ml syringe. Total DNA, RNA and proteins were isolated

according to manufacturer's protocol. The RNA and DNA were dissolved in 30µl of nuclease free water and the concentration as well as the optical density of each sample was measured spectrophotometrically with NanoDrop ND-1000. Proteins extracts were dissolved in 20µl of 1% (w/v) SDS and stored at -20ºC.

Agarose gel electrophoresis: Gel electrophoresis was used to check for genomic DNA contamination and RNA decay. DNA/RNA analysis by agarose gel electrophoresis was carried out using 1% gel (w/v) of SeaPlaque® Agarose (Cambrex, U.K) in 0.5× TBE buffer (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA, pH 7.2). Gels were prepared by boiling the agarose solution in a microwave. After cooling down, SybrSafe™ DNA stain (Invitrogen) was added in a 1:10,000 proportion to allow DNA/RNA visualization and placed in an appropriate cast. Samples were prepared with 6× DNA loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol, in H_2O) and run at 100 V in Life Technologies Horizon gel tanks. A 1 kb molecular weight marker (Invitrogen) was used at a concentration of 0.5 µg per lane as a reference to determine DNA size. To visualize the DNA/RNA, gels were exposed to UV light in a transiluminator ImageQuant 350 GE, HealthCare. Finally the total RNA and DNA was kept at -20ºC until further processing. For long term storage total RNA was kept at -80ºC

2.1.2 Detection of *Babesia bigemina* in ticks by polymerase chain reaction (PCR)

A PCR was performed to detect the presence of *B. bigemina* in the sampled ticks using primers Bbi400F: 5´-AGCTTGCTTTCACAACTCGCC-3´ and Bbi400R: 5´-TTGGTGCTTTGACCGACGACAT-3´ that amplify a 400 bp fragment within the conserved region of the five *rap-1a* paralogous genes (Suarez et al., 2003, Petrigh et al., 2008). These assays were performed with PCR Master Mix, (Promega, Madison, U.S.A.), which is a ready-to-use reaction master mix, containing *Taq* DNA polymerase (capable of adding adenines to the 3' end of the product needed to allow the ligation of the A-tail from the PCR product to the T-overhangs at the end of a T/A vector), dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. The conditions of the PCR were: one amplification round in a final volume of 25 µL including PCR Master Mix 1x, forward and reverse primers to a final concentration of

1µM, 50ng of DNA (40 cycles: 30 s at 94º C, 45 s at 64º C, and 1 min at 72º C; final extension 10 min at 72º C). All the PCR assays were performed in an Applied Biosystems GeneAmp® PCRSystem 2700 apparatus. The results were analysed by electrophoresis, as referred above.

Cloning of PCR products: Cloning PCR products into a stable vector is often desirable for subsequent analysis; in the present case cloning was necessary to confirm obtained DNA sequence. The PCR products of the positive samples were purified with silica spin columns, Invitrogen- PureLink ™ PCR purification kit, for efficient removal of short primers, dNTPs, enzymes, short-failed PCR products, and salts from PCR products. Two amplified products were then cloned using Promega ρ Gem®-T Easy Vector Systems according to the manufacturer's instructions. Briefly, 50ng of the pGEM®-T Easy Vector was incubated, for 1 hour at room temperature, with 2µl of PCR product, 1X Rapid Ligation Buffer and 3 Weiss units of T4 DNA Ligase. Ligation was promptly used or kept at -20ºC.

Transformation: Transformation of plasmid DNA into competent JM109 *Escherichia coli* cells (Promega) was performed by heat shock. 50 µl of competent cells were mixed with 5 µl of ligation reaction and kept on ice for 20 min. The transformation reaction was heat shocked in a water bath at 42 °C for 45 s and placed immediately on ice for 5 min before plating the cells on agar plates.

Selection of transformants: Selection of successful transformants was done by antibiotic selection and the insertion of a PCR product in the vector was assessed by blue/white colony screening. Agar plates were prepared with the appropriate antibiotic (100 µg/ml of ampicillin) and supplemented with 80 µg/ml X-Gal and 0.5 mM of IPTG. Plates were incubated overnight at 37 °C and white colonies were selected for further analysis by colony PCR and plasmid purification.

Colony screening by PCR: To confirm the PCR product integration, single white colonies were picked from the agar plate with a sterile pipette tip and ressuspended in 20 µl of PCR reaction mix, after being patched onto a fresh agar plate for reference. PCR was performed as described previously, using M13F/M13R oligonucleotides, to

determine the presence of an appropriate size insert. PCR products were analyzed on agarose gel and the relevant colonies used to inoculate 5 ml of LB medium. After an overnight incubation at 37 °C, the bacterial culture was used to purify the plasmid DNA.

Plasmid DNA purification: The plasmids DNA were purified using Promega Wizard Plus SV Minipreps DNA, a silica membrane-based system purification system, according to the manufacturer's instructions. Plasmid DNA was eluted in 30µl of nuclease free water and quantified using NanoDrop ND-1000. Plasmids were then sequenced at Secugen, Madrid, Spain and DNA sequence analysed with BioEdit version 7.1.3.0 software.

2.1.3 Complementary DNA (cDNA) library construction and SSH

Total RNA was isolated from nine *R. annulatus* engorged females infected with *B. bigemina* and nine engorged non-infected ticks. RNA quality was checked by gel electrophoresis to confirm the integrity of RNA preparations as described before (section 2.1.1). Two pools corresponding to the infected and uninfected tick populations were made. Poly A^+ RNA was isolated using the FastTrack® 2.0 mRNA Isolation Kit (Invitrogen life Technologies, Carlsbad, CA, USA). The cDNA was synthesized and the SSH library was constructed using the PCR-Select™ cDNA subtraction Kit (Clontech-Takara, Mountain View, CA, USA). Briefly, double stranded cDNA from both groups (infected and non-infected ticks) was RSAI digested. Part of the digested cDNA was ligated with Adapter 1 and part with the Adapter 2R; the rest of cDNA was saved to be used as a driver in hybridization. The forward subtracted library was made by hybridizing adapter ligated cDNA from *B. bigemina* infected ticks as the tester in the presence of uninfected tick cDNA as the driver. This reaction was designed to produce clones that are upregulated in infected ticks.

cDNA library cloning: Differentially expressed cDNAs were subsequently PCR amplified and cloned using a T/A cloning vector. The Advantage ® 2 PCR polymerase mix with TITANIUM *Taq* DNA Polymerase (Clontech, Carlsbad, USA) was used according with the manufacturer instructions and PCR products were used promptly. The TOPO TA Cloning ® Kit for sequencing (Invitrogen) was used which contains the

plasmid vector pCR™4-TOPO®. The manufacturer instructions were followed and the cells used on the transformation were One Shot® TOP10 competent cells. Briefly, transformation of plasmid DNA into competent *E. coli* cells was performed by heat shock. Fifty µl of competent cells were mixed with 4 µl of ligation reaction and kept on ice for 30 minutes. The transformation reaction was heat shocked in a water bath at 42 °C for 30 seconds and placed immediately on ice for 5 minutes before plating the cells on agar plates. Selection of successful transformants was done by antibiotic selection and directly by disruption of the lethal *E. coli* gene, *ccd*B (Bernard et al., 1994). The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligating a PCR product disrupts expression of the *lacZα-ccd*B gene fusion permitting growth of only positive recombinants upon transformation in TOP10 cells. Cells that contain non-recombinant vector are killed upon plating. Therefore, blue/white screening was not required. Agar plates were prepared with the appropriate antibiotic (100 µg/ml of ampicillin) and supplemented with 0.5 mM of IPTG. Plates were incubated overnight at 37 °C and colonies were randomly screened for insertion confirmation analysis by colony PCR using the universal T3/T7 oligonucleotides as described before. PCR products were analyzed on agarose gel as already referred elsewhere and all colonies were used to inoculate 1 ml of LB medium and incubated overnight at 37 ºC and 200 RPM. Bacterial culture was used to purify the plasmid DNA.

Plasmid purification for sequencing: For plasmids purification, illustra plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) was used since it applies a simple plasmid DNA purification protocol involving a modified alkaline lysis procedure and a silica-based membrane to achieve highly efficient plasmid DNA purification (GE Healthcare, 2008). Samples were then centrifuged at 21,000 g during 10 min using a Heraeuspico 17 centrifuge (Thermo Electron Corporation, Marietta, Ohio, USA) and supernatant was discarded. The obtained pellet was further purified using the above described kit according to manufacturer's instructions.

Sequencing and analysis of clones: Eight hundred clones from the SSH library were randomly selected and sequenced at the Department of Genome Sciences, University of Washington, USA. The cDNA Annotation System software (dCAS;

Bioinformatics and Scientific IT Program (BSIP), Office of Technology Information Systems (OTIS), National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA) (http://exon.niaid.nih.gov) (Guo et al., 2009) was used for automated sequence clean up, assembly, blasting against nonredundant sequence database (nr) and databases of tick-specific sequences (http://www.ncbi.nlm.nih.gov and http://www.vectorbase.org/index.php) and Gene Ontology (GO) assignments. Protein ontology was also analyzed using the protein reference database (http://www.proteinlounge.com). Nucleotide sequences were aligned using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) and protein sequences were aligned using the CLUSTAL 2.1 multiple sequence alignment tool (EMBL-EBI; http://www.ebi.ac.uk/Tools/). Phylograms were constructed with protein sequences using the Neighbor-Joining method (Saitou and Nei, 1986) and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and expressed in the units of the number of amino acid substitutions per site (EMBL-EBI; http://www.ebi.ac.uk/Tools/). Unique expressed sequenced tags (EST´s) were deposited in the GenBank with accession numbers JK489362-JK489457.

2.1.4 Confirmation of differential expression by Real time PCR

Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes (Heid et al., 1996, Livak and Schmittgen, 2001). In the present study it was unnecessary to determine the absolute transcript copy number so the relative change in gene expression suffices. Relative quantification will describe the change in expression of the target genes in the infected tick population relative to the reference group, the uninfected tick population.

Total RNA extracted from 18 *R. annulatus* engorged female ticks, 9 of those infected with *B. bigemina* and 9 non-infected was used to construct cDNA using the BioRad® iScript ™ cDNA synthesis Kit. Eight hundred ng of total RNA was used in the synthesis of approximately 1µg of cDNA per sample using the BioRad® iScript ™ cDNA synthesis Kit. The manufacturer's conditions were followed. Primers were designed based on the sequences determined for selected candidate differentially

expressed genes (Table 3) using Primer3 v. 0.4.0 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). It was used 500ng of cDNA on each assay on a total volume of 25µl per sample. The PCR reaction was performed using the iScript SYBR Green RT-PCR Kit (BioRad, Hercules, CA, USA) in a BioRad IQ5 thermo-cycler following the manufacturer's recommendations. The mRNA levels were normalized against tick β-actin and 16S rRNA transcripts using the ddCT method (Livak and Schmittgen, 2001, Schefe et al., 2006). In all cases, the mean of the duplicate values was used and data from infected and uninfected ticks was compared using the Student's t-test (P=0.05).

Table 3: Sequences of primers used for real-time PCR or real time reverse transcription (RT)-PCR.

Gene	Upstream/downstream primer sequence 5´-3´	PCR annealing conditions
Hebrain-like	AGAACTCTCTGCGAGGCTTG TCTTGATGAGATGCGTGAGG	51 ºC/30 s
Mucin-like	ACCGTCGCCTACGATATGAC GGACGTAGAATTCGGGTTCA	51 ºC/30 s
Calreticulin	TGAGAGTCTTGTGGGGAAGG CGTCATCCTCCTTCTTGCTC	51 ºC/30 s
Kunitz-type proteinase inhibitor SHPI-1 chain	CCAAGAGTTGCCAGAGGTTC ACACATTTCAGGTGGTGCAA	51 ºC/30 s
Protease inhibitor carrapatin	ACACTACCCTAAGCGCTGGA TCGGAAAGTAACCCTTGCAG	51 ºC/30 s
Microplusin-like	TCACTTCCAGGAGGTCCATT ACTCTGAGCTCAAGGGCAAG	51 ºC/30 s
GP80 precursor	CCAACTCGCTCAAGACCTTC GTTGGACACCAGCCAGTTCT	51 ºC/30 s
Microplusin preprotein	AGAAGTGCACAGCCCCTAGA GCAACAACTGAACTGCCTCA	51 ºC/30 s
Kunitz-type protease inhibitor 5 (KTPI)	AGCAGCACGTGTCCTTCTTT TGACCAAGACTTGCGAACAG	51 ºC/30 s
Chaperonin, similar	CGCACAAGAATGCTGACAGT CGGGAGGACATTGAGTTTGT	51 ºC/30 s
Von Willebrand factor	GACATGAAAGGAGTGCGTGA AGTGGTTACCAGGGCACTCA	51 ºC/30 s
TROSPA	GTTGGACACCAGCCAGTTCT GCCCAAGCGCATAAATAAGA	51 ºC/30 s
Serum amyloid A	GGCAGGTACTTCCACTGCAT TTGGGTGGTAAAAGCACTCC	51 ºC/30 s
Similar to 5´-3´ exoribonuclease 1	TGGGCTTCTTTTGCAGACTT TCATCCACGTGTGCTCTCAT	51 ºC/30 s
Ricinusin	GAAACTGCAAGCTGTCACCA CTCTTCAGGGTGGGCAGTAG	51 ºC/30 s
Aspartic protease	TCCAGCAATGGTGAATGAAA CAAGGCATGCAAGTCAAGAA	51 ºC/30 s
Subolesin	GATGCACTGGTGACGAGAGA CACAGTCCGAGTGGCAGAT	55 ºC/30 s
β-actin [a]	GACATCAAGGAGAAGCT(TC)TGC CGTTGCCGATGGTGAT(GC)	55 ºC/30 s
16S rRNA [b]	GACAAGAAGACCCTA ATCCAACATCGAGGT	42 ºC/30 s

[a] Described previously by Zivkovic et al.,(2010a).
[b] Described previously by Zivkovic et al.,(2010b).

2.*2 in vivo* gene silencing in ticks by RNA interference

The *Anaplasma* spp., and *Babesia* spp., free *R. annulatus* (Mercedes strain, Texas, U.S.A) and *R. microplus* (Media Joya strain, CENAPA, Mexico) ticks used for the RNAi experiments were obtained from a laboratory colony maintained at the University of Tamaulipas, Mexico. One 3-4 months old male Holstein calf free of babesiosis and anaplasmosis was used to obtain *R. microplus* and *R. annulatus* freshly molted adult female ticks. At day zero (0) the calf was infested with 1 g of each tick specie eggs. Tick-feeding cells (450mmX400mm) (cotton fabric), one per specie, were glued to shaved skin using Pattex® contact glue (Henkel Nederland, Nieuwegein, The Netherlands) and larvae were allowed to feed. At day 15/16 freshly molted adult were collected.

2.2.2 dsRNA synthesis

Gene-specific double-stranded RNA (dsRNA) was synthesized based on identified *R. annulatus* sequences and used to knockdown the expression of selected genes in *R. annulatus* and *R. microplus* ticks injected with dsRNA. Specific primers containing T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´-end were synthesised (Table 4) and RNA was used as template to amplify fragments of interest by RT-PCR which was performed using the Acess RT-PCR Core reagents Promega kit. Reactions included 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1mM MgSO4, 0.1U *Tfl* DNA Polymerase, 0.1U AMV Reverse Transcriptase , the appropriated AMV/*Tfl* reaction Buffer at 1x, 1 µM of each primer and 200ng of template RNA. The final conditions were: First strand synthesis performed at 45 ºC for 45 min then for AMV reverse transcriptase inactivation 3 min at 94 ºC followed by PCR cycling for 40 cycles of 30 s at 94 ºC, 30 s at 55ºC and 90 s at 68 ºC with a final extension step of 7 min at 68º C. All PCR assays were performed in MJ Research PTC-200 Thermo Cycler (GMI Biotech, Minnesota, USA). Amplification results were analysed on a 0.5X TBE, 1.2 % (w/v) agarose gel, purified using the Purelink™ PCR purification kit (Invitrogen) and the MEGAscript RNAi Kit (Ambion, Austin, TX, USA) was used to synthesize dsRNA according to manufacturer's instructions. The resulting dsRNA was purified, quantified by spectrometry and checked on a 0.5X TBE, 1.2 % (w/v) agarose gel.

Table 4: Sequences of primers used for double-stranded RNA synthesis.

Gene	Upstream/downstream primer sequence 5´-3´[a]	PCR annealing conditions	Fragment size (bp)
TROSPA	TGGCGGTGGATATGGAGG CGTTGAGCTCGCCCTTTC	51 ºC/30 s	496
Aspartic protease	CAAGGCATGCAAGTCAAG GTAGGCACACGGCATTCC	51 ºC/30 s	458
Serum amyloid A	GCGATTCGCCCTTGAGCG GTCTACGATTCGCCCTTAG	51 ºC/30 s	324
Kunitz-type protease inhibitor 5 (KTPI)	CGCTGCAGTGCTTCAATCAGCA TTCGCCCTTAGCGTGGTCGC	55 ºC/30 s	248
Ricinusin	ATGAAGCCCACGAAGCCCCG CATGGTGGGCCGCTTCAGGG	55 ºC/30 s	323
Calreticulin	TTCGCCCTTAGCGTGGTCGC GGTCGCGGCCGAGGTACAAA	55 ºC/30 s	526
Subolesin	GACTGGGACCCCTTGCACAGT CGAGTTTGGTAGATAGCACA	55 ºC/30 s	370

*All primers contained T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´end.

2.2.3 dsRNA injection in ticks

Freshly molted *R. annulatus* and *R. microplus* adult female ticks were detached from the bovine host, using fine forceps, observed, cleaned and placed ventral side up on double sticky tape affixed to a 3" x 6" sheet of red dental wax. The ticks are closely positioned together in groups of 10 ticks leaving the body exposed. After, ticks were injected with 0.4 µl of dsRNA (1×10^{11} to 1×10^{12} molecules/µl) in the lower right quadrant of the ventral surface of the tick exoskeleton (de la Fuente et al., 2006b). Thirty female ticks per group were injected using a Hamilton syringe with a 1-inch, 33 G needle. Control ticks were injected with *R. microplus* subolesin dsRNA (positive control) or buffer (10 mM Tris-HCl, pH 7, 1 mM EDTA) alone (negative control). Subolesin dsRNA was synthetized by José de la Fuente (IREC, Spain) following the previously referred guidelines.

Treatment of ticks after injection: Injected ticks were first kept in a recovery plastic container to wait for tick activation and after placed in a humidity chamber (12 hr light: 12 hr dark photoperiod at 22-25 °C and 95% relative humidity) and hold there for 24hr. Ticks were allowed to feed in eight separated circular patches (six test groups and two controls) on a calf that was experimentally infected with $2x10^8$ *B. bigemina* (field

strain from Chiapas, Mexico). Patches used for tick feeding with inner diameter of 150 mm were glued to the shaved back of calf using Pattex® contact glue (Henkel Nederland, Nieuwegein, The Netherlands). Cattle infection was monitored, to ensure that feeding would occur at the peak of host parasitaemia and demonstrated by visual examination of blood smears and PCR. Fifteen male ticks were placed together with each group to allow reproduction. Unattached ticks were removed two days after infestation. All attached ticks were removed after 7 days of feeding and held in a humidity chamber for 4 days to allow ticks to digest the blood meal.

Analysis of tick phenotype after RNAi: Tick phenotype after feeding was evaluated by determining the number of ticks that survived and tick weigh. Tick mortality was evaluated as the ratio of dead ticks to the total number of fed ticks on the calf. To analyze tick mortality, the Chi-square test (P=0.05) was used with the null hypothesis that tick mortality was not dependent on gene knockdown. Ticks were dissected and whole internal organs were stored in RNAlater (Ambion) for total DNA and RNA extraction as described in 2.1.1.

2.2.3 Gene knockdown assessment by real time reverse transcriptase PCR and infection quantification

Gene knockdown of the selected genes was considered by real-time RT-PCR using sequence-specific primers (Table 3) by comparing mRNA levels between dsRNA-injected and control ticks. The *B. bigemina* infection levels were determined by quantitative PCR of the 18S rDNA gene (Genbank AY603402) using primers 5´-AATAACAATACAGGGCTTTCGTCT-3´ and 5´-AACGCGAGGCTGAAATACAACT -3´ and normalizing against tick 16S rDNA gene using the ddCT method (Livak and Schmittgen, 2001, Schefe et al., 2006). The mRNA levels, *B. bigemina* infection in ticks and female tick weight after feeding were compared between dsRNA and saline-injected control ticks by Student's t test (P=0.05).

2.3 Expression and purification of recombinant proteins involved in tick-pathogen interactions

2.3.1 Amplification of selected genes

Four proteins were selected to proceed with the studies, Calreticulin (CRT), TROSPA, serum amyloide A and ricinusin. The CRT and TROSPA sequences were amplified by PCR or reverse transcriptase (RT) PCR respectively using as template either cDNA (synthetized from a RNA pool as described in section 2.1.1) or a RNA pool. For CRT, primers were designed based on the gene sequence of *R. annulatus*, accession number AY395253 (Table 5), ensuring full coding region coverage. Regarding TROSPA, the primers were designed based on the complete coding sequence of the *Ixodes scapularis* TROSPA gene, accession number AY640046 (Table 5). To both forward primers a four base pair sequence (CACC) were included on the 5′ end necessary for directional cloning. The same methodology was attempted for serum amyloid A and ricinusin amplification but all attempts failed. It was decided to proceed with CRT and TROSPA.

Calreticulin: Amplification of the CRT coding region was performed by PCR using the Advantage ® 2 PCR polymerase mix with TITANIUM *Taq* DNA Polymerase (Clontech), under the following conditions: one amplification round in a final volume of 50 µl, including 200 ng of template cDNA and 10 µM of each primer, 2 µM dNTP´s and 1x Advantage 2 PCR Buffer (95 ºC for 2 min, then, for CRT, 40 cycles: 30 s at 94 ºC, 45 s at 55 ºC and 2 min at 72 ºC; final extension 10 min at 72 ºC.

TROSPA: For TROSPA coding sequence amplification a RT PCR was used allowing the cDNA synthesis and PCR amplification to be carried out in the same tube. This assay was performed using the Acess RT-PCR Core reagents (Promega). The reaction included 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1mM $MgSO_4$, 0.1U *Tfl* DNA Polymerase, 0.1U AMV Reverse Transcriptase , the appropriated AMV/*Tfl* reaction Buffer at 1x, 1 µM of each primer and 200 ng of template RNA. The final conditions were: First strand synthesis performed at 45 ºC for 45 min then for AMV reverse transcriptase inactivation 3 min at 94 ºC, followed by PCR cycling for 40 cycles of 30 s at 94 ºC, 30 s at 67 ºC and 90 s at 68 ºC, with a final extension step of 7 min at

68ºC:). All PCR assays were performed in MJ Research PTC-200 Thermo Cycler (GMI Biotech, Minnesota, USA). PCR produts were analysed by electrophoresis, as referred in section 2.1.1. The positive PCR products were purified using the illustra GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions. Purified samples were sequenced at Stab Vida (Almada, Portugal) and further analysed.

Table 5: Primer sequences used for coding region amplification

Gene	Upstream/downstream primer sequence 5´-3´[a]	PCR annealing conditions	Fragment size (bp)
Calreticulin	CACC AT GCG GCT TCT CTG CAT TTT G CAG TTC TTC GTG CTT GTG GTC	55 ºC/45 s	1235bp
TROSPA	CACC ATGGCGGCTA TGGAGGCTATG ACTTCCAGCGGCGCTCTGGTC	67 ºC/30 s	496bp

2.3.2 Expression of recombinant proteins

Recombinant proteins were expressed fused to histidine (his) tag, to increase solubility and allow purification. For recombinant expression in *E. coli* system, Champion^{TM} pET101 directional TOPO® Expression Kit (Invitrogen Life Technologies, Carlsbad, California, USA) was used. This expression kit uses a highly efficient 5-min cloning strategy to insert a blunt end PCR product into a vector with no requirement of post-PCR procedures or restriction enzymes (Invitrogen, 2010). Recombinant calreticulin was obtained as it will be specified in the next sections. For recombinant TROSPA expression the plasmid vector construction was synthesized by GenScript (Piscataway, NJ, USA) and protein was expressed under similar conditions as rCRT by José de la Fuente and collaborators in IREC, Spain.

Cloning and transformation: Briefly, the purified PCR products, previously obtained, were initially cloned into plasmids pET101/D-TOPO vectors using the above described kit according to manufacturer's instructions. 10 µl of pET TOPO® (Invitrogen Life Technologies, Carlsbad, California, USA) construct, previously obtained, was mixed with 50 µl of the *E. coli* OneShot® cells (Invitrogen Life Technologies, Carlsbad,

California, USA), and then incubated in ice during 30 min, followed by incubation for 30 s at 42ºC and further incubated in ice. Afterwards, 250 µl S.O.C. medium were added to the previous sample and incubated at 37ºC, 200 RPM during 1 hour. After that period, 200 µl of the sample were used to seed LB-agar/ampicillin (100 µg/ml) Petri-dishes. Plates were incubated overnight at 37 °C and colonies were randomly screened for insertion confirmation analysis by colony PCR using the universal T3/T7 oligonucleotides.

Screening of the transformed colonies: Cell colonies were analyzed by PCR to confirm plasmids incorporation. Six colonies were picked up and individually suspended into 12 µl of water. Kit GoTaq® (Promega, Madison, Wisconsin, USA) was used. The PCR conditions were as followed: 94ºC for 10 min to lyse cells and inactivate nucleases, then 35 cycles: 94ºC for 3 min, 53ºC for 30 s and 72ºC for 1 min, followed by 72ºC for 10 min, in MJ Research PTC-200 Thermo Cycler (GMI Biotech, Minnesota, USA)

Plasmid purification for sequencing: For plasmids purification, illustra plasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) was used as already described in (section 2.1.3) and samples were then sequenced in Stab Vida (Almada, Portugal).

Expression: Plasmids, previously purified, were inserted in BL21 Star™ (DE3) One Shot *E. coli* cells according to manufacturer's instructions. The entire transformation was used to inoculate 10 ml of LB/ampicillin (100 µg/ml) and was incubated overnight at 37ºC and 200 RPM. Cells were incubated at 37ºC and 200 RPM, split into two 5 ml cultures and one was induced at an Abs 600 nm (optical density) of 0.5-0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). To determine the optimal induction time, 500 µl aliquot from each culture have been removed every hour for 5 hours. Samples were centrifuged at 21,000 g for 1 min, the supernatant was discarded and pellets were frozen at -20ºC. For large-scale expression a 20 ml culture was used to inoculate 500 ml of LB/ampicillin (100µg/ml) and induced with the appropriate amount of IPTG once Abs 600 nm reached 0.8.

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis): A common method for protein separation by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature proteins. According to Laemmli method, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze cellular lysate and confirm the presence of recombinant proteins. Cellular lysates were typically separated using a 15% (w/v) polyacrylamide gel. Gels were made with 30% acrylamide-bis solution (BioRad) in plastic casting cassettes (Invitrogen) with a 5% (w/v) stacking gel layer above the 15% (w/v) resolving gel, to help load and focus the protein samples before separation. Protein samples were prepared in 6× SDS-PAGE loading buffer (2× SDS-PAGE loading buffer: 20% (v/v) glycerol, 2.5% (w/v) SDS, 0.05% (w/v) bromophenol blue, 0.2 M Tris-HCl pH 6.8, 10% DTT in H2O) and boiled for 5 minutes at 100°C to promote denaturation. Electrophoresis was performed in Mini-protean Tetra Cell (Bio-Rad, Hercules, California, USA) with 1× SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). Voltage was set initially at 100 V and then increased to 180 V once the samples reached the resolving gel, until the protein dye front reached the bottom of the gel. In each gel, a low molecular marker (Amersham^{TM} LMW calibration kit for SDS electrophoresis, GE Healthcare, Buckinghamshire, UK) was run alongside with the protein samples to estimate their apparent molecular weight. After electrophoresis, protein gels were stained with Coomassie stain solution (2.5% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid in H_2O) for one hour at room temperature, shacking. Gels were then immersed in several changes of destaining solution (10% methanol (v/v), 10% (v/v) glacial acetic acid in H_2O) until a good resolution was observed. After destaining, gels were washed in distilled water and photographed.

Western-Blotting: WB is a technique that identifies proteins which have been separated according to their size by gel electrophoresis, using specific antibodies. The blotting membrane, usually made of nitrocellulose or PVDF (polyvinylidene fluoride), binds to proteins. With this method, the polyacrylamide gel is placed over the membrane and the application of electrical current forces proteins to move from gel to membrane, where they adhere and can be subsequently linked to a specific antibody. This binding

can then be visualized using a second antibody which recognizes the first one, developing a visual signal in the presence of the appropriate substrate (Abcam, 2012). For WB analysis, proteins were transferred from a SDS-PAGE gel to a nitrocellulose membrane (Trans-Blot® Transfer Medium pure cellulose membrane (0.45 μm), Bio-Rad, Hercules, California, USA) using Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad) with transfer buffer (20% (v/v) methanol, 25 mM Tris, 192 mM glycine in H2O) for 1 hour at 360 mA or overnight at 90 mA. After the transfer, the membrane was stained in Ponceau Red 0.2% (w/v) diluted in acetic acid 3% (v/v) to ensure that the transfer was successful. Membrane was cut into strips and after washing in water, membranes were blocked with 5% (w/v) powdered milk (Biorad) in TTBS buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, 0.1% (v/v) Tween-20) for 1 or 2 hours at room temperature or overnight at 4ºC, shaking. Subsequently, the strips were washed 3 times with 2 ml of TTBS for 15 min. Strips were incubated in 1 ml of primary antibody (anti-histidine antibodies solution (1:5000) (Anti-His (C-term) antibody, Invitrogen Life Technologies, Carlsbad, California, USA) for 1 hour or overnight at 4ºC shaking. After incubation with the primary antibody, membranes were washed three times for 5-10 minutes and incubated with 1ml of the secondary antibody, anti-mouse antibody solution (1:10000) (Anti-Mouse whole IgG molecule - Alkaline Phosphatase, Sigma-Aldrich, St. Louis, Missouri, USA) for 1 hour and the wash procedure was repeated. 1.5 ml of revelation buffer (AP Color Development Buffer, Bio-Rad, Hercules, California, USA) was used to develop the visual signal. The reaction was stopped by adding water and keeping the membrane in the dark.

Purification of recombinant proteins: A pellet from a 500 ml culture was resuspended in 30 ml lysis buffer (100 mM Tris pH 8.0, 0.2% (v/v) Triton X-100) frozen at -20 ºC and thawed at 42 ºC. Lysis was performed by sonication (Ultrasonic Homogenizer Power Supply 4710 Series, Cole Parmer, Illinois, USA) with an amplitude of 80%, a frequency of 20 kHz and cycles of 10 s/min for a total of 5 min. Subsequently, samples were centrifuged for 15 min at 13000 g, pellet was suspended in 1 ml of PBS and supernatant stored. These samples were used to perform a SDS-PAGE and WB, as described before in order to determine whether the proteins were present in the soluble or insoluble fractions of lysate. To perform the purification of recombinant proteins a 5ml HisTrap HP prepacked with precharged Ni Sepharose™ column (GE Healthcare,

Buckinghamshire, UK) associated with fast performance liquid chromatography (System Pump-500, GE Healthcare, Buckinghamshire, UK) was used. Prior to purification, samples were diluted in binding buffer (20 mM Na_2PO_4, 500 mM NaCl and 30 mM imidazole, pH 7.4). Purification protocol was as follows:

1. Column was washed with 3 volumes of distilled water and equilibrated with 5 volumes of binding buffer at a flow-rate of 5 ml/min;

2. Soluble fractions were applied with a pump and contaminants were removed as the column was washed with binding buffer until absorbance reached a steady baseline;

3. Recombinant proteins bound to the column were eluted using a one-step gradient at a flow rate of 2 ml/min, 5 volumes of elution buffer (20 mM Na_2PO_4, 500 mM NaCl and 500 mM imidazole, pH 7.4) were generally used;

4. Peak fractions were pooled, flow through was collected and peek fractions were pooled and its purity assessed by SDS-PAGE;

Different imidazole concentrations (binding buffer 10mM, 20mM and elution buffer 100mM, 200 mM, 300 mM, 500 mM) as well as pH values (pH 7.4, 8.0 and 8.8) in the buffers were tested in order to select optimal purification conditions. When necessary purified samples were concentrated using an Amicon Ultra-0.5 centrifugal filter with a cut off of 10,000 kDa.

Determination of protein concentration: Protein concentration was determined using Bradford reagent (Sigma) according to the manufacturer's instructions. Assays were performed in 96 well plates and standard BSA curves were prepared with concentrations ranging from 0 to 2 mg/ml. All samples were done in triplicates and analysed at a wavelength of 595 nm, using the software Microplate manager 4.0 (BioRad) in a microplate reader (BioRad model 550). The standard values were used to plot a calibration curve of absorbance versus BSA concentration, and the regression line was used to determine the concentration of the different samples.

2.4 Production and characterization of antibodies against tick proteins involved in tick-pathogen interactions

2.4.1 Poly and monoclonal antibodies production

Immunization: 4-6 weeks old female BALB/c mice were immunized intraperitoneally with 20 μg of recombinant protein emulsified with incomplete Freund's adjuvant (Sigma-Aldrich), every 2-3 weeks until a satisfactory antibody titer was reached. After 3 to 5 immunizations, blood samples were obtained from mice for serum titer determination by enzyme-linked immunosorbent assay (ELISA) (technique described in the next section). Once the titer was high enough cell fusion was performed. Three days before the cell fusion, mice were boosted with the antigen and then euthanized by cervical dislocation for spleen removal. Mice total blood as also collected in order to obtain antiserum, which is extremely rich in antibodies against the recombinant antigens resulting in a positive control that can be used in further analyses and trials.

Antibody titer monitoring of mouse serum using ELISA: Antibody production was determined for mice injected with recombinant proteins. Approximately 50 μl of blood was removed from the tail of the mice to be analyzed; the serum was obtained by centrifugation at 2000 *g* for 10 min. A high binding ELISA plate (Costar® 96-Well EIA/RIA plate) was incubated overnight at 4ºC with 0.1 μg of recombinant protein per well, diluted in 100 μl PBS. Negative control consisted in wells incubated with only with PBS. After antigen incubation, the plate was blocked with 200 μl of 5% (w/v) milk (BioRad) at room temperature for one hour and washed three times Tris Buffered Saline (25 mM Tris, 150 mM NaCl, 2 mM KCl) / 0.05% (v/v) Tween 20 (TBST). First antibody (serum) was incubated for one hour at 37 ºC; A two fold serum dilution was added to the wells containing the antigen, starting at 1:200 reaching a minimum dilution of 1:16000. Plates were then washed three times with TTBS. Secondary antibody; Anti-Mouse Polyvalent Immunoglobulins AP conjugated (Sigma), was diluted 1:10,000 in TTBS supplemented with 0.1% BSA (w/v) (Sigma), added to all wells and incubated for one hour at 37 ºC. After washing 5× with TTBS, plates were incubated with 1 mg/ml of p-nitrophenil phosphate in substrate buffer (100 mM glycine, 1 mM MgCl2, 1 mM ZnCl2, pH 10.4) at room temperature in the dark, until the color yellow is developed. Plates were

then read in a microplate reader (BioRad) at a wavelength of 405 nm and analyzed with Microplate manager 4.0 software (BioRad). Antibody production was considered positive when a titre greater than 1:3200 was obtained.

Generation of hybridomas: Sp2/0 myeloma cells were cultured in GIBCO® DMEM (Dulbecco's Modified Eagle Medium) with 10% GIBCO® FBS (fetal bovine serum containing 8-azaguanine (Sigma-Aldrich) to ensure their sensitivity to hypoxanthine-aminopterin-thymidine (HAT) selection used after the cell fusion has occurred. A week before the cell fusion, the 8-azaguanine was removed from the medium. Single spleen cells from immunized mice were then fused with the myeloma cells by co-centrifugation in the presence of polyethylene glycol (PEG) using classic Cell Fusion/Hybridoma production technique. Briefly, after being removed from the mouse, the spleen was immersed in DMEM, in a petri dish, and the cells were removed by injecting medium inside the spleen, repeatedly until all the cellular content was all in suspension. The spleen cell suspension was then centrifuged for 10 minutes at 560 g and cells resuspended in 10 ml of DMEM. Ten µl of resuspended cells were used to determine cell density and viability with Trypan blue (Sigma-Aldrich) in an improved Neubauer haemocytometer counting chamber. In parallel, the cellular density of the Sp2/0 cells was also determined and both cellular suspensions were mixed in a 1:10 proportion of Sp2/0: spleen cells. The Sp2/0 and spleen cells suspension was then centrifuged at 560 g for 10 minutes and the supernatant discarded. PEG-DMSO (Sigma-Aldrich) was slowly added and gently mixed with the cell pellet, leaving it to rest for 1 minute. The PEG-DMSO was then diluted first by slowly adding and gently mixing 1 ml of DMEM and then by an additional volume of 20 ml of DMEM over 5 minutes time. The suspension was centrifuged at 560 g for 10 minutes and the cell pellet was ressuspended in 25 ml of complete DMEM medium supplemented with 10% FBS and HAT 1× (Sigma – Aldrich) and plated on four 96-well plates. Twenty four hours after the cell fusion, 100 µl of complete DMEM medium was added to each well and plates were left to grow at 37 °C in a 5% CO_2 incubator.

Hybridomas culture: Hybridomas were cultured initially in complete DMEM medium (10% FBS and HAT 1×) at 37 °C in a 5% CO2 incubator. When expanded from

24-well plates to 25 cm2 tissue culture flasks, hybridomas were cultured either in DMEM with 5% FBS and HAT 1× or DMEM with 5% FBS with HT (hypoxanthine-thymidine). All culture work was done under sterile conditions in a laminar flow hood.

Screening for antibody producing hybridomas: The principle for creating an antibody secreting hybridoma is based on the cellular fusion between antibodies producing spleen cells, with limited life spam, with cells derived from an immortal tumor of lymphocytes that do not synthesize immunoglobulin, myeloma cells. The resulting hybridoma not only is capable of producing and secreting antibodies but also of unlimited growth. As the myeloma cell line is defective in the enzyme hypoxanthine guanidine phosphoribosyltransferase (HGPRT), they die when cultured in DMEM supplemented with HAT (aminopterin blocks the main DNA synthesis pathway and the alternative pathway that requires the use of exogenous hypoxanthine depends on the enzyme HGPRT). Only hybrids between myeloma and spleen cells are capable of surviving in HAT supplemented medium. At day 10, supernatants of the wells containing hybridomas (previously screened under the microscope) were tested by ELISA. Assay plate was prepared as described previously, including a well with no antigen (PBS only) to account for non-specific reaction of the primary antibody (antiserum). Four different controls were performed against the recombinant protein: a positive control using the antiserum diluted 1:1000; and three negative controls (primary antibody (antiserum) with no secondary antibody; no primary antibody with secondary antibody; no primary or secondary antibodies). Hybridoma supernatant was used as primary antibody. The hybridomas selected by ELISA were then expanded to 24-well plates and subsequently to 25 cm^3 tissue culture flasks and supernatants tested again by ELISA and also by WB as described above but using supernatants as primary antibody. For storage supernatants were filtered using a 0.22 μm syringe filter (Carl Roth GmbH) and kept at 4ºC or -20ºC.

Hybridomas storage: For cryopreservation, hybridoma cultures were centrifuged at 560 g for 10 minutes Cell pellet was resuspended in 1ml of FBS with 4% DMSO (dimethyl sulfoxide), aliquoted in cryovials and stored immediately at -80 °C for 24 hours before being transferred to liquid nitrogen tanks for long term storage. To reestablish frozen cultures, cells were defrosted rapidly and ressuspended in 20 ml of DMEM

medium with no serum or HAT, and centrifuged at 1200 x *g* for 10 minutes. The pellet was then resuspended in 5 ml of complete medium and incubated at 37 ºC and 5% CO_2.Cell growth was monitored at regular intervals.

2.4.2 Immunolocalization assays

The obtained antibodies were used to detect the presence of the native antigens on different tick tissues by western blot and immunofluorescence. Tick specimens used in these assays belonged to populations reared for previous studies.

Western blotting: Cattle tick proteins were isolated using a standard lysis buffer (50mM Tris pH8.4; 50 mM NaCl; 0.5% (v/v) Triton x-100; 0.02% (w/v) sodium azide). First, ticks were dissected and tissues separated in pools of tick midgut, ovaries and salivary glands. The soft tissues of an entire *R. annulatus* tick were also used. Lyses buffer (200µl) was added to each tissue pool and samples were homogenized using a 21G × 1½" (0.8 × 40mm) needle and 1ml syringe. Samples were frozen and thaw three times to promote protein release from cells. Samples were centrifuged for 5 min at 7500g at 4ºC and supernatant was placed in a new 1.5 ml eppendorf tube. Proteins were quantified with Bradford reagent as described in section 2.3.2, Protein extracts (30 and 50 µg per well) were separated in a SDS-PAGE gel and stained with coomassie (section 2.3.2) prior to WB assays to visualize the extract. Western blot was performed as described previously in section 2.3.2. Mice antiserum was tested as well as produced antibodies.

Indirect immunofluorescence: Cattle tick tissues (guts, salivary glands, and ovaries) were dissected in ice- cold PBS buffer from individual engorged females. All tissues were washed in PBS giving special attention to guts samples from which the luminal contents were carefully removed, and remaining tissue was gently washed from the host blood excess in the same buffer. Tissues were either used immediately after dissection, or stored at -80°C in RNAlater (Ambion, Austin, TX, USA). To prepare samples for indirect fluorescent microscopy, dissected tissues were either paraffin-embedded to obtain thin tissue sections or used directly.

Preparation of paraffin-embedded tissue sections: tissues were place in small plastic cassettes, fixed in 3.7% formaldehyde for at least 2 hours and then dehydrated in ascending ethanol dilutions as follow: one hour in 70% ethanol, one and a half hour twice, at 95% ethanol, one hour to one and a half hour three times in absolute ethanol and two hours twice in xylol. Tissues were after infiltrated in paraffin (Histosec, Merck) at 65º C for two hours twice. After cooling, tissues were sectioned using a Leica SM2010 R sliding microtome. 2 µm sections were placed in a glass slide and allowed to dry. The tick tissue was then deparaffinizate dewaxed in xylene and hydrated in decreasing ethanol concentrations: 15 minutes in xylol twice, followed by two minutes in absolute ethanol, two minutes in 95% ethanol, two minutes in 70% ethanol and finally retained in distilled water.

Whole tissue preparation: Dissected salivary glands from partially fed cattle tick females were applied to microscope glass slides pre-coated with 0.01% poly-l-lysine and fixed with 4% formaldehyde in PBS for 30 minutes at room temperature.

Processing after tissue placement in the glass slide was the same for both preparation types. To stain the internal cells the tissue was permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 minutes and washed three times with excess PBS before blocking overnight with 3% (w/v) BSA at 4ºC. After washing the slides again with excess PBS, primary antibody (1:100) diluted in blocking solution, was applied and the slides incubated for 1 hour at 37ºC. The slides were then washed three times with PBS and the appropriate secondary antibody, Alexa Fluor 488 (green)-conjugated anti-mouse (Molecular Probes), diluted (1:100) in blocking solution was applied and slides incubated for 1 hour at 37ºC. After a final PBS wash, a drop of ProLong® Gold Antifade Reagent with 4′, 6′-diamidino-2-phenylindole (DAPI) (Invitrogen) was placed over the tissues and then slides were sealed with a coverslip. Slides were kept in a moist dark box until microscopic analysis to prevent drying and fluorescence fading. Tick tissues sections were visualized under a Nikon eclipse 80*i* fluorescence microscope with appropriate filters. Entire salivary glands were viewed under a Zeiss LSM 710 confocal microscope.

2.4.3 Tick capillary feeding

The *R. microplus* ticks (Media Joya strain, CENAPA, Mexico), used on the capillary feeding assays, were reared in University of Querétaro, Mexico. Originally, these tick strains were collected from naturally infested cattle. One 3-4 months old male Holstein calf free of babesiosis were used to obtain *R. microplus* female ticks. The calf was infested in day 0 (zero) with larvae hatched from 0, 5 g of *R. microplus* eggs. Partially engorged *R. microplus* females were recovered manually from calves at days 20/21.

Infected and uninfected bovine blood: To obtain infected *B. bigemina* blood one 6 month old Holstein calf was splenectomized and 2 weeks later, intravenously inoculated with cryopreserved $2x10^8$ *B. bigemina* (Chiapas strain). The infection was monitored by animal temperature, hematocrit and blood smears. Thin capillary blood, taken from bovine ears, smears were fixed with methanol, stained with 10% Giemsa for 20–30 minutes, and examined at ×800–1000 magnification under oil immersion. Approximately 10 000 red blood cells (RBCs) were screened for infection. Once the infection reached 0.7% (infected RBCs per 100 RBCs counted) 500 ml of blood was collected from the jugular vein to a collection set which includes a needle with needle cover, a needle guard, a tube holder, a 500 mL whole blood collection bag with 70 mL citrate phosphate dextrose anticoagulant. The uninfected bovine blood was obtained in the same way from a healthy Holstein calf free of babesiosis and anaplasmosis.

Antibodies: Polyclonal antibodies were used in artificial feeding assays and not the previously obtained monoclonal antibodies. Serum obtained from immunized mice with rCRT was used to supplement bovine blood, as well as PBS immunized mice serum. Approximately 20µl of serum was presented to each tick. Purified immunoglobulin's G (IgGs) from serum of rTROSPA immunized rabbits, IgGs anti-subolesin and IgGs pre-immunization was also used. These antibodies were produced in collaboration with José de la Fuente (IREC, Spain): for each tick protein, three New Zealand white rabbits (*Oryctulagus cuniculus*) were subcutaneously injected at weeks 0, 3 and 6 with 0.5 ml containing 50 µg protein in Montanide ISA 50V adjuvant (Seppic, Paris, France). Blood was collected before injection and two weeks after last immunization to prepare

preimmune and immune sera, respectively. Serum aliquots were kept at 4 °C for immediate use or at -20 °C for long-term storage. IgGs were purified from serum samples using the Montage Antibody purification kit and spin columns with PROSEP-A Media (Millipore, MA, USA) following manufacturer's recommendations. Approximately 100 µg of IgGs were offered to each tick.

Artificial feeding: Partially engorged cattle tick *R. microplus* females were recovered manually from calves 20/21 days after the beginning of infestation. Ticks were then cleaned, weighed and fixed on expandable polystyrene trays (214x114mm), with double face tape. Females with damaged mouthparts and/or weighing more than 60 mg or less than 20 mg were discarded. These trials were performed in a controlled temperature and relative humidity room. Temperature was set at 26-28 °C and relative humidity above 85%. Females were divided in experimental groups, each formed by 15 individuals, and fed for 28 h as follows: group fed with bovine blood only; group fed with bovine blood supplemented with mice serum or IgGs pre-immunization; groups fed with bovine blood supplemented with anti-rCRT serum, anti rTROSPA IgGs and anti subolesin IgGs. Similar groups were organized using 0.7% *B. bigemina* infected blood. Microhematocrit capillary tubes (75 mm x1.0 x1.5 mm) without anticoagulant were filled with bovine blood and placed over the ticks' mouthparts promoting feeding. Capillary tubes were repositioned and/or replaced when necessary or every 3 hours. After the feeding process, the females were detached from the double face tape and weighed again to determine blood ingestion and placed in clear individual 2ml pierced microtubes and maintained in an incubator at 27 ºC and 85% humidity to allow oviposition. Total tick weight and egg weight were determined and examined independently. Comparisons on weight increment and oviposition weight between groups were determined by Student's t test with unequal variance. Statistical significance was designated a priori as a P-value 0.05.

PCR to determine pathogen infection levels in ticks: In order to quantify pathogen infection levels, 10 ticks per group were taken three days after feeding and their internal tissues dissected to obtain total RNA and DNA using Tri reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's recommendations. *B. bigemina* infection levels were determined by quantitative PCR as already described in section 2.1.4 *B.*

bigemina infection levels (arbitrary units) in ticks was compared between ticks fed with blood supplemented with antibodies against the selected recombinant proteins and control ticks fed with bovine blood supplemented with pre-immunization antibodies by Student's t test with unequal variance (P=0.05).

Evaluation of tick mRNA levels of genes encoding for antigens: The *calreticulin* and *trospa* mRNA levels were characterized in 10 ticks per group by real-time RT-PCR as described previously in section 2.1.4 using the oligonucleotide primers described in table 3. Real-time RT-PCR was done using the same conditions as described in section 2.1.4. The mRNA levels were normalized against tick 16S rRNA using the comparative Ct method. Normalized mRNA levels were compared between ticks fed with blood supplemented with antibodies against the selected recombinant proteins and control ticks fed with bovine blood supplemented with pre-immune antibodies by Student's t test with unequal variance (P=0.05).

Results

The results presented are subdivided according to important milestones accomplished during the project. The first three subchapters address the identification of differentially expressed genes in *Babesia bigemina* infected cattle female ticks. The following subchapter includes the functional analysis of selected genes. Subchapters 3.5 and 3.6 concerns to the expression of recombinant proteins followed by the production of polyclonal antibodies raised against the expressed recombinant proteins. The last two subchapters present the results of immunolocalization trials and tick artificial feeding which contributes to the characterization of the selected antigens.

3.1. Identification of differentially expressed genes in *Rhipicephalus annulatus* female ticks

Suppression subtractive hybridization was used to identify *R. annulatus* tick genes differentially expressed in response to *B. bigemina* infection. In this study 80 *Rhipicephalus annulatus* ticks were used to extract total RNA as well as DNA and the presence of *Babesia bigemina* was evaluated. Sixty female *R. annulatus* ticks were fed on a *B. bigemina* infected calf and 20 similar ticks were fed on a healthy, free of babesiosis and anaplasmosis, calf. RNA quality was checked in an agarose gel as showed in figure 8.

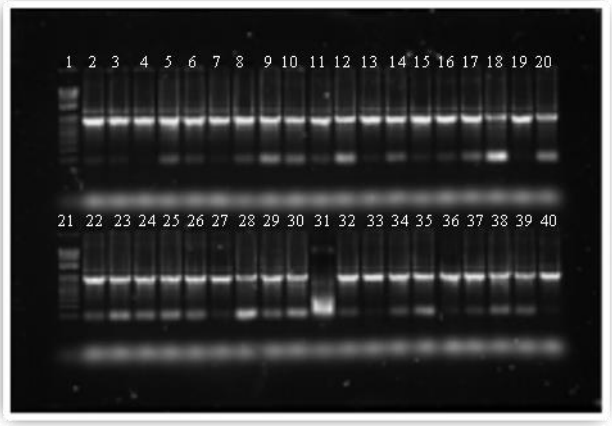

Figure 8: Example of the total RNA extracted using TRI-Reagent SIGMA®.
Samples were electrophoresed on a 1% Agarose gel/SYBRSafe, 0.5 x TBE. Samples 1 to 38, the 1st well on the upper and lower row corresponds to the ladder, 1 kb DNA plus Invitrogen®.

To confirm the infection by *Babesia bigemina* in the ticks it was used a PCR assay to amplify a 400-bp fragment within the *B. bigemina* conserved region of the *rap-1a*

paralogous gene (Petrigh et al., 2008). From the 60 *R. annulatus* ticks fed on a *B. bigemina* infected calf, PCR only confirmed the presence of the parasite in 9 of the tick samples (Figure 9). One of the factors that could have been responsible for this low number of positives is the quality of the DNA used; other is the one-step PCR here used. Some of the described analysis carried on to confirm *B. bigemina* presence applies nested PCR which can be more sensible for low levels of parasitaemia (Figueroa et al., 1993, Guerrero et al., 2007). As positive control was not available, the obtained fragment was cloned and sequenced. Sequence showed a 99% identity to the *B. bigemina* strain S2P RAP1 beta-3 (RAP-1 beta-3) gene, accession number AF017296.

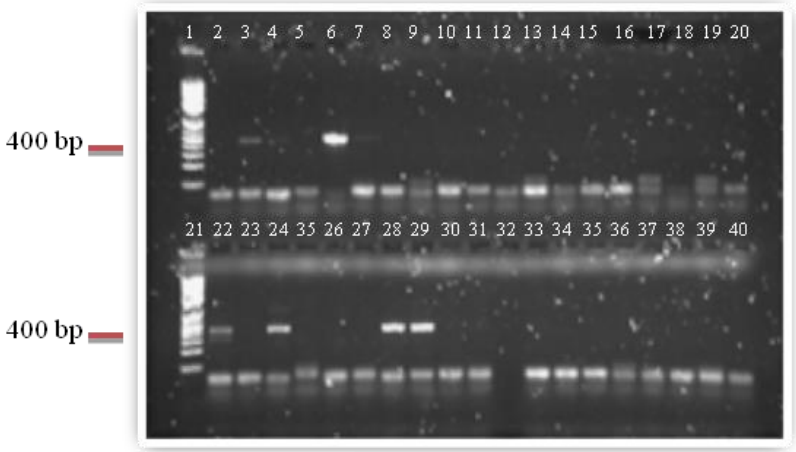

Figure 9: Detection of *Babesia bigemina* in *Rhipicephalus annulatus* ticks by PCR.
Gel electrophoresis of PCR results using the protocol from (Petrigh et al., 2008) with the modified annealing temperature to 64ºC. Samples were electrophoresed on a 1% Agarose/SYBRsafe gel, 0.5 x TBE. Samples 1 to 38, the 1st well on the upper and lower row corresponds to the ladder, 1 kb DNA plus Invitrogen®

The infected and non-infected *R. annulatus* ticks were used to generate two total RNA pools corresponding to the uninfected (driver population) and the infected population (tester population) to use in the SSH. RNA pools showed a concentration of 10.1 mg /ml and 8.7 mg /ml and, after RNA poly A^+ isolation, 5.4 mg/ml (uninfected) and 5.3 mg /ml (infected). Quality of the pools was analysed in an agarose gel. From these, 20 µg were used in a forward subtraction step resulting in SSH library enriched for differentially regulated cDNAs in the infected population. The sequences obtained were cloned using Invitrogen TOPO TA Cloning kit and resulting colonies were screened (Figure 10).

Figure 10: Cloning of SSH library sequences.
Gel electrophoresis of a colony PCR using T3 and T7 primers showing the presence of inserts in the clones. Lane 1: 100bp Ladder DNA Fermentas™; Lane 12: PCR control; All samples were electrophoresed on a 1% Agarose/SYBRSafe gel 0.5x TBE

Eight hundred randomly selected SSH library clones were sequenced and analyzed. After eliminating clones with poor quality sequences, 752 sequences (average length ± SD, 562±297 bp) were assembled into 96 unigenes (87 contigs and 9 singlets) representing unique expressed sequence tags (ESTs) (Additional table in appendix 1). On average, the number of sequences per unigene was 8.3, which suggested a low diversity in the dataset. Assembled ESTs resulted in 41 (43%) ESTs with unknown function or without identity to sequence databases. Significant identity to genes with functional annotation was confirmed for one (1%) *Babesia* T2Bo hypothetical protein EST, 5 (5%) ESTs related to the vertebrate host and 49 (51%) ESTs with similarity to tick sequence databases. Of the 49 ESTs with similarity to tick sequences, 7 (14%) corresponded to protease inhibitors (Additional table in appendix 1). Molecular function gene ontology (GO) assignments showed that the obtained tick sequences encoded for proteins with different functions such as cell structure, defense, transport, signal transduction and regulation, synthesis, energy metabolism and enzymatic processes (Figure 11).Terms used on figure 11 were selected to simplify the results due to the narrow information that GO terms can sometimes provide. The categories were established using the information available, for example, at protein reference database.

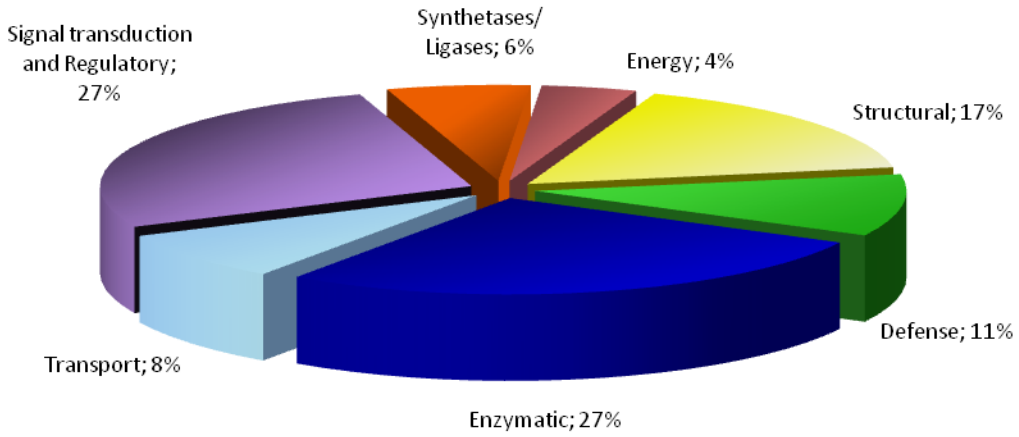

Figure 11: Functional grouping of tick genes differentially expressed in *B. bigemina*-infected *R. annulatus*, based on Gene Ontology (GO) molecular function assignments

3.2 Differential gene expression in *Babesia bigemina*-infected *Rhipicephalus annulatus* ticks

Based on the ontological analyses, sixteen candidate genes with putative functions in tick-pathogen interactions were selected for validation of SSH results by real time PCR. Out of the sixteen genes analysed, five were confirmed as differentially expressed in *B. bigemina*-infected ticks, showing expression differences statistically significant. For the other 11 genes, mRNA levels were not significantly different between the infected and non-infected *R. annulatus* ticks tested. Accordingly to SSH results, genes encoding for putative TROSPA, calreticulin, ricinusin and serum amyloid A proteins were significantly overexpressed in infected ticks. The gene encoding for putative Kunitz-type protease inhibitor 5 (KTPI) identified by SSH to be up regulated was shown by real time to be down-regulated in infected ticks (Figure 12).

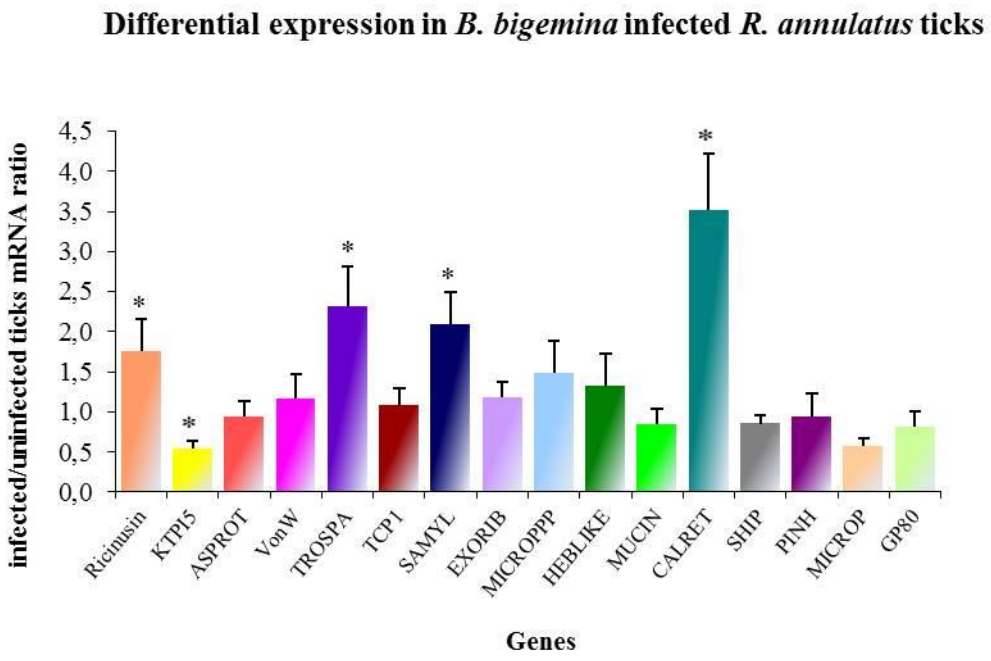

Figure 12: Differential gene expression in *B. bigemina* infected *R. annulatus* ticks.

The mRNA levels of selected differentially expressed genes were determined by real-time RT-PCR using the RNA pools prepared from uninfected and infected ticks. The mRNA levels were normalized against tick β-actin and 16S rRNA transcripts using the ddCT method. In all cases, the mean of the duplicate values was used and data from infected and uninfected ticks were compared using the Student's t-test ($P<0.05$).

3.3. Sequence analysis of tick genes differentially expressed in response to *Babesia bigemina* infection

Additional sequence analysis was conducted on *R. annulatus* ESTs confirmed as differentially expressed in response to *B. bigemina* infection. *R. annulatus* EST68 (GenBank accession number JK489429) sequence analysis showed that TROSPA is a highly conserved gene in ticks, with 78% (128/165 amino acids) homology between *R. annulatus*, *I. ricinus*, *I. scapularis* and *I. persulcatus* protein sequences (Figure 13).

Figure 13: TROSPA multiple amino acid sequence alignment.

Sequence aligned included *R. annulatus* EST68 (JK489429), *I. ricinus* (ABU43150), *I. scapularis* (AAO43095), and *I. persulcatus* (BAK09229). Asterisks denote amino acids conserved in all sequences analyzed.

For calreticulin, *R. annulatus* EST21 (JK489382) showed a 99% (226/229 nucleotides) identity to *R. microplus* calreticulin precursor (AF420211) 3´-end coding region with 97% (73/75 amino acids) homology to protein COOH-terminal region. The *R. annulatus* EST84 (JK489445) showed the highest homology (97%; 97/100 amino acids) to *I. ricinus* ricinusin (ABB79785) and to a lesser extend to *I. scapularis* microplusin preprotein-like (AAY66716) and *R. microplus* microplusin (AAO48942) (Figure 14). The *R. annulatus* EST81 (JK489442) showed 46-49% homology to previously reported *Ornithodoros parkeri* Cooley, 1936 (EF633889) and *I. scapularis* (XM_002407273, XM_002416454) serum amyloid A protein-like sequences. Four different *R. annulatus* ESTs (EST24, EST25, EST28, EST29, EST42) showed homology to Kunitz-type protease inhibitors (Additional table in appendix 1), with a maximum 62% (32/52 amino acids) homology to *I. scapularis* serine proteinase inhibitor (XM_002434100).

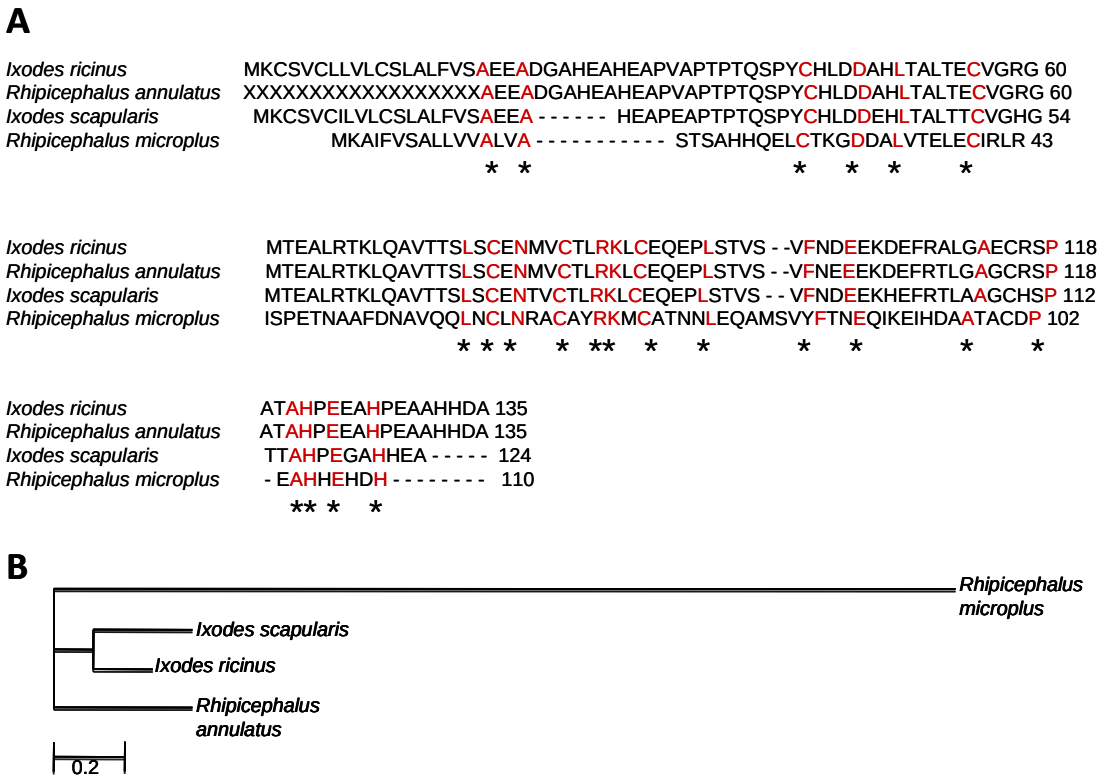

Figure 14: Analysis of ricinusin ortholog sequences.
(A) Amino acid sequence alignment of *R. annulatus* EST84 (JK489445), *I. ricinus* ricinusin (ABB79785), *I. scapularis* microplusin preprotein-like (AAY66716) and *R. microplus* microplusin (AAO48942). Asterisks denote amino acids conserved among all sequences analyzed. (B) Unrooted phylogram inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

3.4 Functional analysis of tick genes differentially expressed in response to *B. bigemina* infection

The five genes confirmed to be differentially expressed in infected ticks (TROSPA, calreticulin, ricinusin, serum amyloid A, and KTPI), together with one gene which mRNA levels were similar between infected and uninfected ticks (aspartic protease) and subolesin, previously shown to be involved in tick innate immunity, were selected for functional studies using dsRNA-mediated RNAi in both *R. annulatus* and *R. microplus*. Figure 15 shows the obtained fragments for double-stranded RNA (dsRNA) synthesis and figure 16 A and B, the obtained dsRNA.

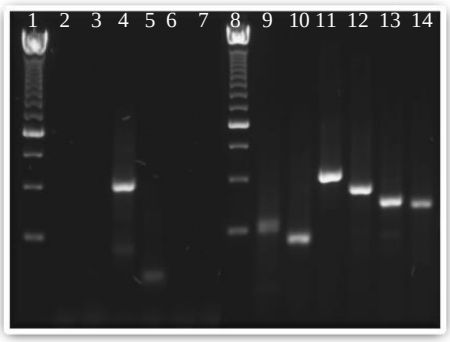

Figure 15: Amplification of fragments of interest for dsRNA synthesis.
Lane 1: 250bp ladder Invitrogen®; Lane 2: ricinusin; Lane 3: KTPI5; Lane 4: aspartic protease; Lane 5: TROSPA; Lane 6: serum amyloid A; Lane 7: calreticulin; Lane 8: 250bp ladder Invitrogen®; Lane 9: ricinusin; Lane 10: KTPI5; Lane 11: aspartic protease; Lane 12: TROSPA; Lane 13: serum amyloid A; Lane 14: calreticulin. Samples from lane 2 to 7 were obtained performing traditional PCR using cDNAs. From lane 9 to 14 fragments were obtained by RT-PCR using RNA as template. All samples were electrophoresed on a 1% Agarose/SYBRSafe gel 0.5x TBE.

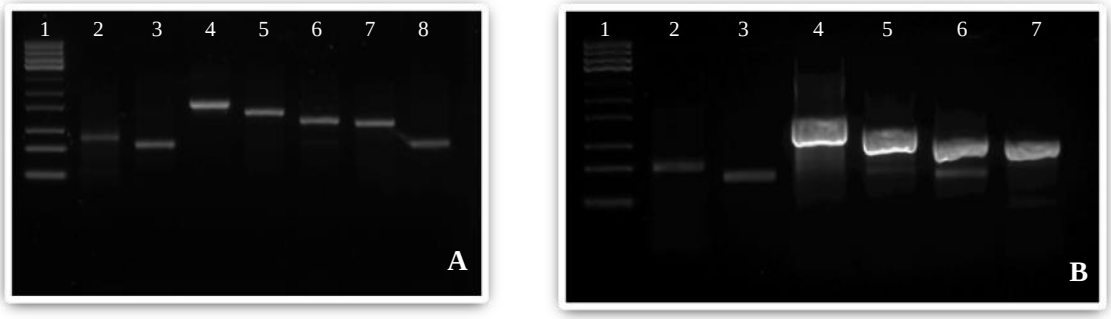

Figure 16: Synthesis of dsRNA.
(A) Purified RT-PCR products. Lane 1: Fermentas MassRuler express reverse DNA ladder; Lane 2: ricinusin; Lane 3: KTPI5; Lane 4: aspartic protease; Lane 5: TROSPA; Lane 6: serum amyloid A; Lane 7: calreticulin; Lane 8: ricinusin; B) Synthetized dsRNA. Lane 1: Mass ruler express reverse DNA ladder; Lane 2: ricinusin; Lane 3: KTPI5; Lane 4: aspartic protease; Lane 5: TROSPA; Lane 6: serum amyloid A; Lane 7: calreticulin. Electrophoresis were done in a 1.2% Agarose/SYBRSafe gel 0.5x TBE.

The effect of gene knockdown on *B. bigemina* infection levels, tick weight and mortality was evaluated. Gene knockdown after dsRNA-mediated RNAi was demonstrated for all genes in *R. annulatus*, while in *R. microplus* the silencing of subolesin and aspartic protease genes was not demonstrated (Tables 6 and 7). Knockdown of TROSPA, serum amyloid A and ricinusin significantly reduced *B. bigemina* infection levels by 83%, 66% and 32%, respectively in *R. annulatus* when compared to control ticks (Table 6). In *R. microplus*, knockdown of TROSPA and serum amyloid A also reduced pathogen infection levels by 70% and 86%, respectively while calreticulin knockdown resulted in 73% lower infection levels when compared to controls (Table 7). Subolesin knockdown did not affect *B. bigemina* infection levels in *R. annulatus* ticks (Table 6)

Table 6: *Babesia bigemina* infection levels after gene knockdown by RNA interference (RNAi) in *Rhipicephalus (Boophilus) annulatus* ticks.

Gene	*N*	Gene silencing (%; Mean ± S.D.)	*B. bigemina* infection levels (Mean ± S.D.)	Test/control (Mean ± S.D.)
TROSPA	7	60 ± 30	6.55E-06 ± 1.13E-07	0.17 ± 0.03[a]
Aspartic protease	20	88 ± 13	3.75E-05 ± 9.33E-06	0.97 ± 0.06
Serum amyloid A	16	59 ± 30	1.33E-05 ± 4.10E-06	0.34 ± 0.04[a]
KTPI	8	100 ± 0	1.84E-05 ± 1.91E-06	0.49 ± 0.14
Ricinusin	17	65 ± 25	2.64E-05 ± 5.94E-06	0.68 ± 0.03
Calreticulin	8	68 ± 33	2.46E-05 ± 5.37E-06	0.66 ± 0.26
Subolesin	9	61 ± 31	2.86E-05 ± 2.14E-06	0.74 ± 0.31
Control	19	---	3.86E-05 ± 7.07E-06	---

Table 7: *Babesia bigemina* infection levels after gene knockdown by RNA interference (RNAi) in *Rhipicephalus (Boophilus) microplus* ticks.

Gene	*N*	Gene silencing (% Mean ± S.D.)	*B. bigemina* infection levels (Mean ± S.D.)	Test/control (Mean ± S.D.)
TROSPA	9	97 ± 6	2.77E-05 ± 1.07E-05	0.30 ± 0.07[a]
Aspartic protease	5	0 ± 0	ND	ND
Serum amyloid A	10	48 ± 31	1.31E-05 ± 2.40E-06	0.14 ± 0.00[a]
KTPI	14	93 ± 17	9.60E-04 ± 6.36E-04	11.35 ± 8.97
Ricinusin	12	94 ± 8	1.81E-04 ± 5.87E-05	2.07 ± 1.01
Calreticulin	14	93 ± 6	2.47E-05 ± 4.67E-06	0.28 ± 0.10[a]
Subolesin	9	0 ± 0	ND	ND
Control	17	---	9.09E-05 ± 1.58E-05	---

Thirty female ticks per group were injected with double stranded (ds) RNA or saline control. Ticks were allowed to feed in eight separated patches on a calf experimentally infected with B. bigemina. All attached ticks (n = 5-17) were removed after 7 days of feeding and held in a humidity chamber for 4 days to allow ticks to digest the blood meal. Gene knockdown was analysed by real-time reverse transcription (RT)-PCR by comparing mRNA levels between dsRNA-injected and control ticks. The B. bigemina infection levels were determined by quantitative PCR of the 18S rRNA gene and normalized against tick 16S rRNA using the ddCT method ($2^{-(CT^{target} - CT^{16s})}$). The mRNA levels and B. bigemina infection in ticks were compared between dsRNA and saline-injected control ticks by a Student's t test ([a] $P < 0.05$). ND, not determined, because gene knockdown was not demonstrated.

The knockdown effect of selected genes on tick weight and mortality was as well determined and statistically analyzed. KTPI knockdown reduced female tick weight in both *R. annulatus* and *R. microplus* (Table 8). For other genes such as TROSPA, ricinusin and calreticulin, gene knockdown resulted in lower tick weight when compared to controls in one of the tick species (Table 8). The effect of subolesin knockdown was only characterized in *R. annulatus* and resulted in the reduction of tick weight when compared to controls (Table 8). Tick mortality was not affected in dsRNA-injected ticks when compared to controls. For all genes and in both tick species, *R. annulatus* and R. *microplus*, the null hypothesis was accepted (P>0.05), suggesting that none of the studied genes had a role on tick survival.

Table 8: Female tick weight after gene knockdown by RNA intereference (RNAi) in *Rhipicephalus (Boophilus) annulatus* and *Rhipicephalus microplus* ticks.

Gene	Tick weight Mean ± S.D. (mg)	
	R. annulatus	***R. microplus***
TROSPA	95 ± 90	30 ± 16[a]
Aspartic protease	72 ± 53	ND
Serum amyloid A	148 ± 118	115 ± 122
Kunitz-type protease inhibitor 5 (KTPI)	37 ± 11[a]	22 ± 11[a]
Ricinusin	49 ± 42[a]	110 ± 110
Calreticulin	37 ± 26[a]	146 ± 136
Subolesin	21 ± 14[a]	ND
Control	89 ± 84	62 ± 48

Thirty female ticks per group were injected with double stranded (ds) RNA or saline control. Ticks were allowed to feed in eight separated patches on a calf experimentally infected with B. bigemina. All attached ticks were removed after 7 days of feeding, weighed and held in a humidity chamber for 4 days to allow ticks to digest the blood meal. Tick mortality was evaluated as the ratio of dead ticks to the total number of placed ticks on the calf. Female tick weight after feeding was compared between dsRNA and saline-injected control ticks by a Student's t test ([a]P < 0.05). ND, not determined because gene knockdown was not demonstrated.

3.5 Amplification of calreticulin and TROSPA cDNA fragment

Tick antigens previously identified as involved in pathogen infection were selected for further characterization. Based on obtained ESTs from SSH, PCR or reverse-transcriptase PCR technique was used to amplify the coding regions correspondent to

CRT and TROSPA (respectively), in order to allow the posterior recombinant proteins production.

As it can be observed in figure 17 A and B, amplified fragments showed the expected size and were further purified and sequenced. Figure 38 in the appendix section (Appendix 2) shows the CRT nucleotide sequence obtained with 1100 bp instead of the expected 1235 bp due to limitations of the sequencing method.

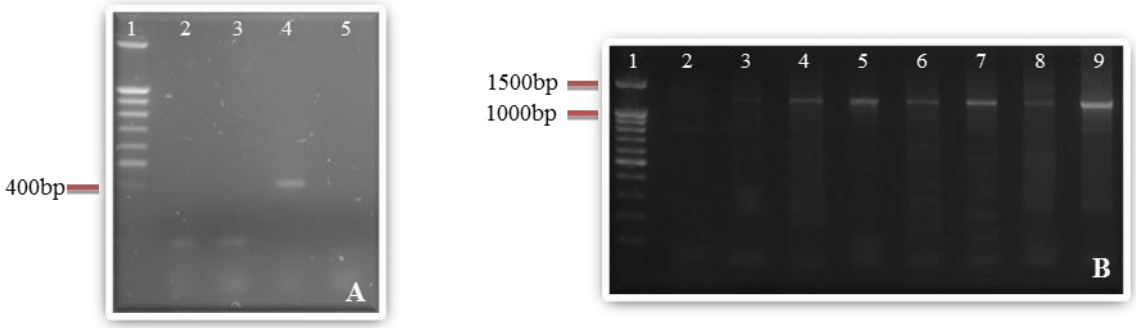

Figure 17: Amplification of fragments of interest by RT-PCR (A) and PCR (B).
Samples were electrophoresed on a 1% Agarose/SYBRSafe gel, 0,5x TBE. A) Lane 1: Promega®100bp ladder; Lane 2 and 3: failed amplifications; Lane 4: Fragment of about 400bp; Lane 5: negative control; B) Lane 1: Promega® 100bp ladder; Lane 2-8: fragments of about 1250bp.

A comparison with other tick available CRT sequences in Genbank (accession numbers stated in the table) was done and the results are shown in table 9. It is to note that in the obtained CRT sequence is missing about 135bp so the identity percentage presented should be regarded merely as informative. A very high identity is observed between tick species due to the high conservation observed in the CRT protein. The described CRT protein sequences from *R. annulatus* (accession number AAR29939), *R. microplus* (AGK88372), *Oryctolagus cuniculus* Linnaeus, 1758 (AAA31188), *Mus musculus* Linnaeus, 1758 *(*AAH03453) and *Bos taurus* Linnaeus, 1758 (BAB86913) was also compared to advise about the limitations of using this protein as immunization antigen (Figure 39 in appendix 2). It was perceived that CRT from *R. annulatus* and *R. microplus* had a similarity to all *O. cuniculus*, *M. musculus* and *B. taurus* of around 68%. This high conservation between species poses a problem that may be surpassed by the use of adjuvants during immunizations and more rounds of immunization.

Table 9: Sequence identity between different available CRT nucleotide sequences and the obtained CRT sequence expressed as percentages.

	CRT sequence	*R. microplus* AY395254	*R. annulatus* AY395253	*R. sanguineus* AY395275	*I. scapularis* AY690335	*A. americanum* U07708	*D.variabilis* AY241957.
CRT sequence	100	93,5	94,2	87,3	77,6	82,8	86
R. microplus AY395254	100	99,1	93,5	76,9	86,3	89,9	
R. annulatus AY395253	100	93,3	77	86,2	89,8		
R. sanguineus AY395275	100	76	85,5	89,5			
I. scapularis AY690335	100	77	76,8				
A. americanum U07708	100	87,8					
D.variabilis AY241957	100						

In the case of TROSPA, the obtained fragment did not corresponded to the available sequence of *I. ricinus* neither to the EST68 (JK489429) obtained previously. Despite several attempts it was not possible to amplify the correct nucleotide sequence and thus, a synthesized plasmid construction containing an insert correspondent to the *I. ricinus trospa* sequence (AY640046) was used.

3.6 Expression of recombinant proteins calreticulin and TROSPA

To express recombinant proteins pET101/D-TOPO® (Invitrogen Life Technologies, Carlsbad, California, USA) plasmid was used as vector. Figure 18 shows the correct insert size in the vector of CRT fragment.

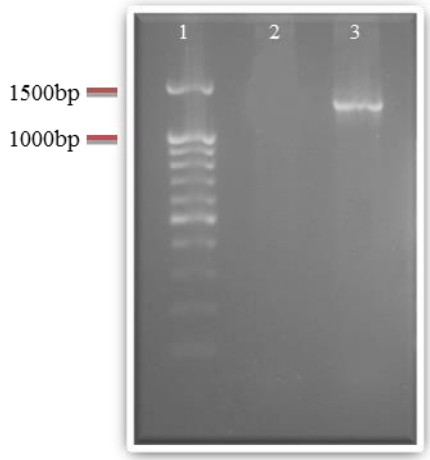

Figure 18: Confirmation of correct fragment size cloning in pET101/D-TOPO® plasmid.
Lane 1: 100bp ladder (Promega); Lane 2: Negative PCR control; Lane 3: One Shot® TOP10 E. coli cells mple. Samples were electrophoresed on a 1% Agarose/SYBRSafe gel, 0.5x TBE.

The level of rCRT expression was first evaluated by SDS-PAGE, as shown in figure 19. An evident difference in expression of a band between 45 kDa and 66 kDa in the IPTG induced culture was not perceived so a Western-Blot procedure using first an anti-histidin primary antibody was performed in order to confirm the expression of CRT. Figure 19 shows a unique band obtained in induced fraction of time points 1 and 2, which correspond to 1 and 2 hours of induction, respectively, there were no bands in the other time points (T3 to T5).

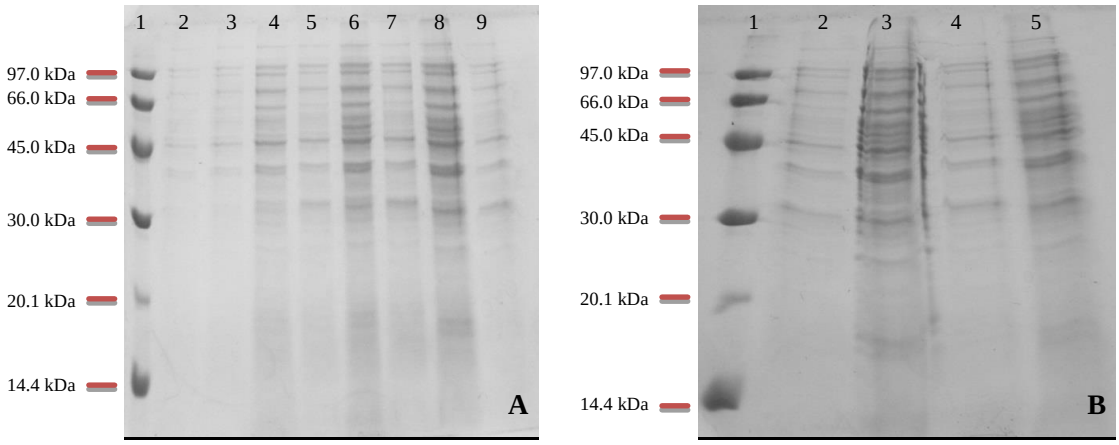

Figure 19: Expression of rCRT.
Whole cell lysates of rCRT expression induced by IPTG and non-induced at different time points were separated on a 12,5% SDS-PAGE gel Coomassie blue-stained. (A) Lane 1: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); Lane 2: T0 induced; Lane 3: T0 non induced; Lane 4: T1 induced; Lane 5: T1 non induced; Lane6: T2 Induced; Lane 7: T2 non induced; Lane 8: T3 induced; Lane 9: T3 non induced; (B) Lane 1 Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare): Lane 2: T4 induced; Lane 3: T4 non induced; Lane 4: T5 induced; Lane 5: T5 non induced.

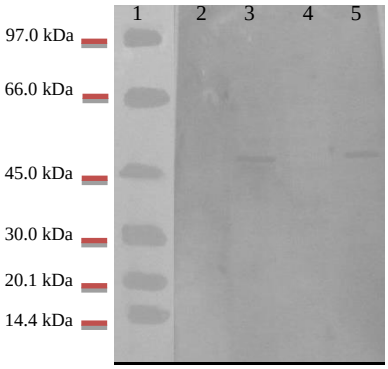

Figure 20: rCRT purification probed by WB with an anti-His antibody.
Lane 1: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); Lane 2: Time point 1hour non induced; Lane 3: Protein induction at 37ºC for 1 hour; Lane 4: Time point 2 hours non induced; Lane 5: Protein induction at 37ºC for 2 hours;

Recombinant CRT was purified using HisTrap HP prepacked with precharged Ni Sepharose™ column (GE Healthcare, Buckinghamshire, UK) associated with fast performance liquid chromatography (System Pump-500, GE Healthcare, Buckinghamshire, UK). Apparent purity level of the obtained samples was determined by SDS-PAGE (Figure 21). A total of 0.3 mg of rCRT was obtained from a 500 ml cell culture.

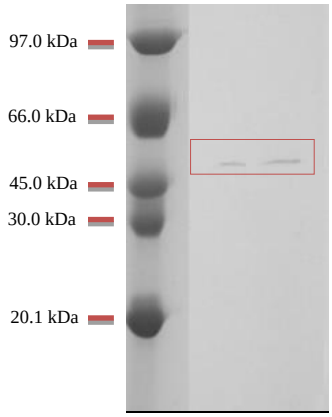

Figure 21: Purified recombinant calreticulin.
Coomassie blue-stained SDS-PAGE gel. 1µg of recombinant CRT was electrophoresed on a 12.5% SDS-PAGE gel. Lane 1: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); Lane 2 and Lane 3: Purified rCRT

For TROSPA protein expression a recombinant construct was synthetized (GenScript,Piscataway, USA) from the sequence AY640046 and further expressed and purified by José de la Fuente and collaborators (IREC, Spain). From an initial 500 ml

Bl21 *E. coli* cell culture it was obtained 0.5 mg/ml of rTROSPA. The purity was estimated by densitometry analysis of the Coomassie blue-stained SDS-PAGE gel (Figure 22).

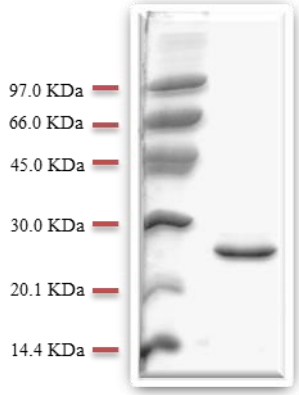

Figure 22: Purified recombinant TROSPA.
Coomassie blue-stained SDS-PAGE. 2µg of recombinant TROSPA was electrophoresed on a 12.5% SDS-PAGE gel. Lane 1: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare); Lane 2: rTROSPA

3.7 Production of monoclonal antibodies

For the generation of monoclonal antibodies against rCRT and TROSPA, female BALB/c mice were immunized with 20µg of each recombinant protein, every two to three weeks. During the immunization process, blood samples were collected and the serum titer determined for each mouse by ELISA, using the recombinant proteins as antigen. After four immunizations, ELISA assays showed the immune response against rTROSPA was strong since O.D. values where the triple of the negative control (Figure 23). Due to a weaker immune response to rCRT, one more immunization was done. After a final immunization boost animals were sacrificed and two separate cell fusions were performed. Seven days after cell fusion hybridomas started to appear on both cases.

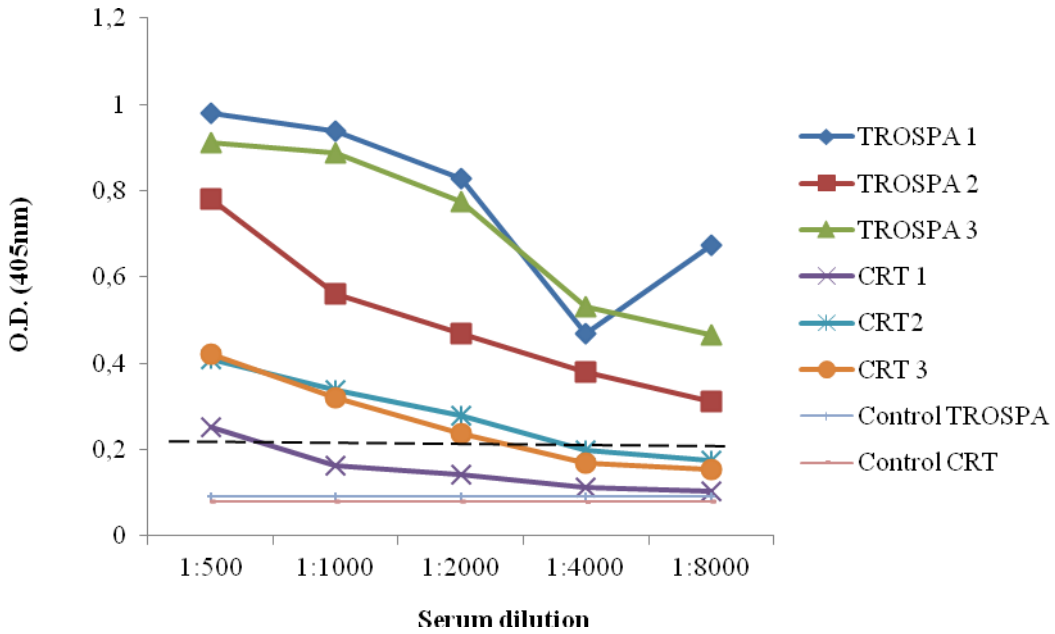

Figure 23: Serum titer for each mouse after 4 immunizations with recombinant proteins.
Threshold level represents the triple of the negative control OD value

Supernatants from the emerging hybridomas were tested by ELISA and the positive ones were collected and expanded initially to 24 well plates and, subsequently, to 25 cm^3 culture flasks after new screening by ELISA (Table 10). Once in flasks, hybridomas were left to grow for approximately 7 days and supernatants were tested simultaneously by ELISA and Western Blotting against the respective recombinant and also against *R. annulatus* protein extracts, to assess their specificity.

Table 10: Hybridomas screened by ELISA. Supernatants from hybridoma cultures were considered positive when showing OD values above the triple of the negative control value

	96 wells plates		**24 wells plates**		**25cm^3 flasks**	
	Tested	**Positive**	**Tested**	**Positive**	**Tested**	**Positive**
rTROSPA	384	57	11	8	8	5
rCalreticulin	384	38	24	11	11	6

Despite the high values (compared to the control) of O.D. 405nm displayed by most antibodies generated against TROSPA, only three recognized the recombinant protein by Western Blotting (Figure 24). From the six antibodies that recognized rCRT in

ELISA assay, four (3C8; 2D12; 1D11; 2A6) recognized the correct protein in WB analysis (Figure 25).

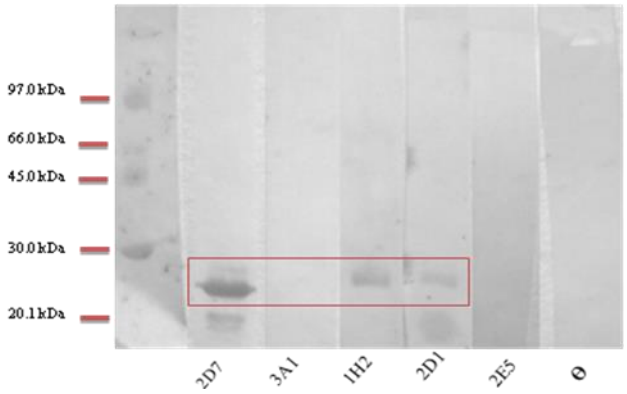

Figure 24: Screening of supernatants from hybridoma cultures by WB against rTROSPA.
2 µg of rTROSPA was electrophoresed on 12, 5% SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with neat hybridoma supernatants as primary antibody and Anti-mouse IgG whole molecule antibody as secondary antibody. A negative control was performed using serum from PBS immunized mice (θ). Ladder: Low Molecular Weight calibration kit for SDS electrophoresis (GE Healthcare)

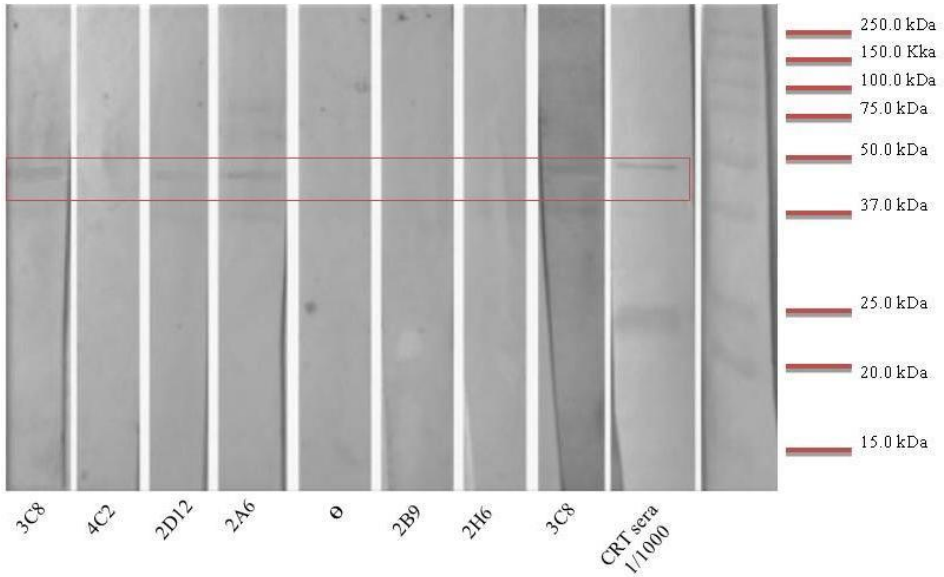

Figure 25: Screening of supernatants from hybridoma cultures by WB against rCRT.
2 µg of rCRT was electrophoresed on 12, 5% SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with neat hybridoma supernatants as primary antibody and a Anti-mouse IgG whole molecule antibody as secondary antibody. A positive control was performed, using anti-rCRT serum diluted 1:1000 as primary antibody and a negative control (ɵ) serum from PBS immunized mice. Ladder: Biorad prestained precision plus protein Dual Xtra.

Supernatants were filter sterilized and stored for further use. Based in these results, the best supernatants were selected for further WB analysis using tick protein extracts investigating not just for the recognition of the native protein but also for the specificity of the antibodies produced.

3.8 Imunolocalization

3.8.1 Western-blot analysis

Immune serum and monoclonal antibodies anti-TROSPA and anti-CRT were used in WB to determine the localization of these proteins in tick tissues. Internal organs of *R. annulatus* were used as well as tissues separated in midgut, ovaries and salivary glands. Protein extracts were obtained from tissue pools as can be observed in figure 26.

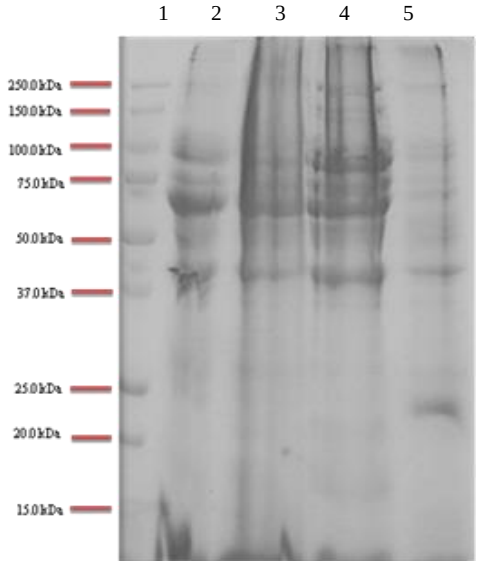

Figure 26: SDS-PAGE gel with *R. annulatus* protein extracts.
Tick internal tissues were homogenized with lysis buffer, frozen and thaw for protein release. Lane 1: Biorad prestained precision plus protein Dual Xtra ladder; Lane 2: *R. annulatus 50* µg protein extract; Lane 3: *R. annulatus* midgut 50 µg protein extract; Lane 4: *R. annulatus* ovaries 50 µg protein extract; Lane 5: *R. annulatus* salivary glands 30 µg protein extract.

To investigate the specificity of rTROSPA and rCRT immunized mice serum and 2D7 and 3C8 supernatants were probed against *R. annulatus* 10 µg of protein extracts (Figure 27). Both serum and supernatants recognized native protein.

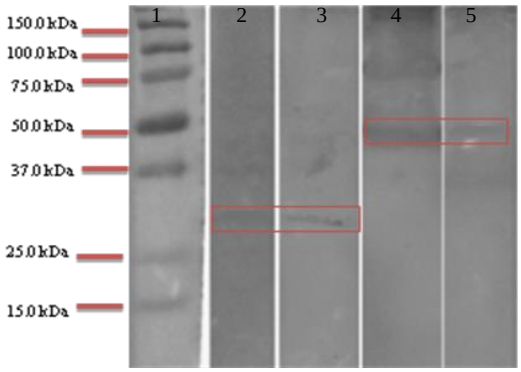

Figure 27: WB analysis of specificity of rTROSPA and rCRT immunized mice serum and 2D7 and 3C8 supernatants against *R. annulatus* protein extracts.
Tick tissues were homogenized with lysis buffer, frozen and thaw for protein release. 10µg of protein extract was electrophoresed on 12, 5% SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with neat hybridoma supernatants as primary antibody or anti recombinant proteins mice serum and anti-mouse IgG whole molecule antibody as secondary antibody. Lane 1: Biorad prestained precision plus protein Dual Xtra ladder; Lane 2: Whole tick tissue lysate incubated with anti rTROSPA 1/1000 mice serum; Lane 3: Whole tick tissue lysate probed with 2D7 supernatant; Lane 4: Whole tick tissue lysate probed with anti rCRT 1/1000 mice serum; Lane 5: Whole tick tissue lysate probed with 3C8 supernatant.

Several *R. annulatus* lysates corresponding to different tissues were used for WB analysis. Supernatant 3C8 was probed against these extracts and as it can be observed in figure 28 only on protein extract corresponding to the entire tick a band of approximately 46 kDa was recognized. This result can be due to the quantity of native protein present in the tissue.

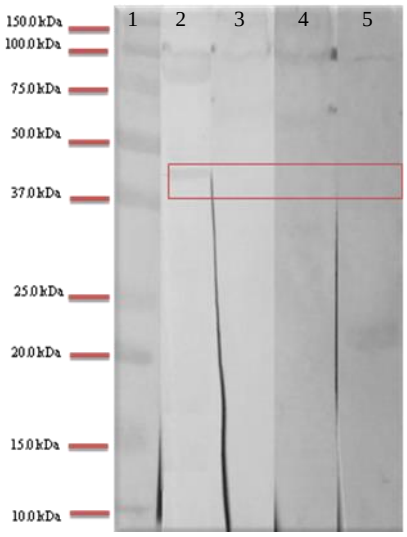

Figure 28: WB analysis of specificity of rCRT 3C8 supernatant against different *R. annulatus* protein extracts.
Tick tissues were homogenized with lysis buffer, frozen and thaw for protein release and electrophoresed on 12, 5% SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with neat hybridoma supernatants as primary antibody or anti rCRT mouse serum and anti-mouse IgG whole molecule antibody as secondary antibody. Lane 1: Biorad prestained precision plus protein Dual Xtra ladder; Lane 2: Whole tick tissue lysate; Lane 3: Midgut protein extract; Lane 4: Ovaries protein extract;

In this case, anti-TROSPA antibodies, serum recognized a single band of the expected size in all the tick lysates with exception to salivary glands. On the other hand the supernatant 2D7 recognized a single band of the expected size in the protein extract from midgut, ovaries and salivary glands (Figure 29). Since there is no control on the amount of native protein in the tick extracts, WB analysis may sometimes differ. These results show that equally serum and supernatants of both antigens can be used for further assays like IFA.

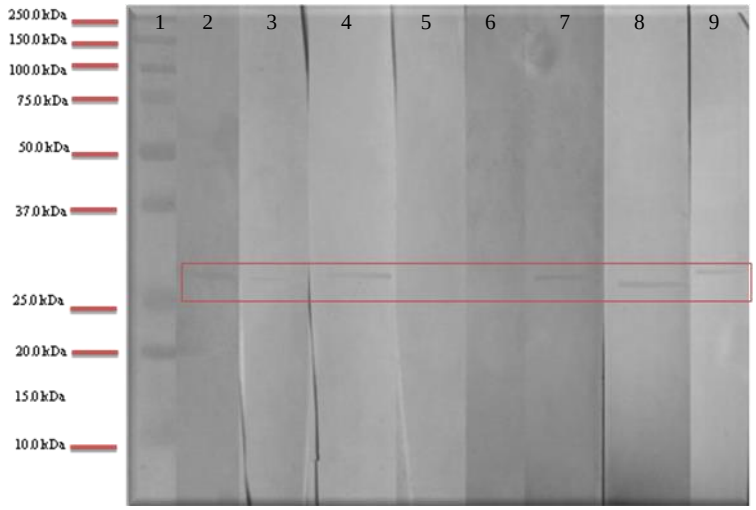

Figure 29: WB analysis of specificity of rTROSPA immunized mice serum and 2D7 supernatant against different *R. annulatus* protein extracts.
Tick tissues were homogenized with lysis buffer, frozen and thaw for protein release and electrophoresed on 12, 5% SDS-PAGE gels and transferred to a nitrocellulose membrane for WB with antiTROSPA mouse serum or neat hybridoma supernatants as primary antibody and anti-mouse IgG whole molecule antibody as secondary antibody. Lane 1: Biorad prestained precision plus protein Dual Xtra ladder; Lane 2: Whole tick tissue lysate incubated with anti rTROSPA mouse serum; Lane 3: Midgut protein extract probed with anti rTROSPA mouse serum; Lane 4: Ovaries protein extract probed anti rTROSPA mice serum; Lane 5: Salivary glands proteins probed with anti rTROSPA mouse serum; Lane 6: WB negative control; Lane 7: Midgut protein extract probed with 2D7 supernatant; Lane 8: Ovaries protein extract probed with 2D7 supernatant; Lane 9: Salivary glands proteins probed with 2D7 supernatant.

3.8.2. Immunofluorescence assays

Produced antibodies were also used to recognize tick native proteins (CRT and TROSPA) by immunofluorescence in different *R. microplus* tissues (Figures 30 - 33). Histological cuts were first stained in typical hematoxylin and eosin to visualize cells (Figure 41 in appendix 3). Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Fixed paraffin-embedded tissue sections of entire salivary glands were first used to infer about the presence of TROSPA protein in tick tissues and after in infected and uninfected

salivary glands (Figure 33). These preliminary assays showed that anti- CRT antibodies recognized tick native CRT in salivary glands but without a clear localization (Figure 30 A-F). Antibodies recognized native antigen in tick midgut (Figure 30 G-I) and in the ovaries a disperse signal within an immature egg can be observed (Figure 30 J-L).

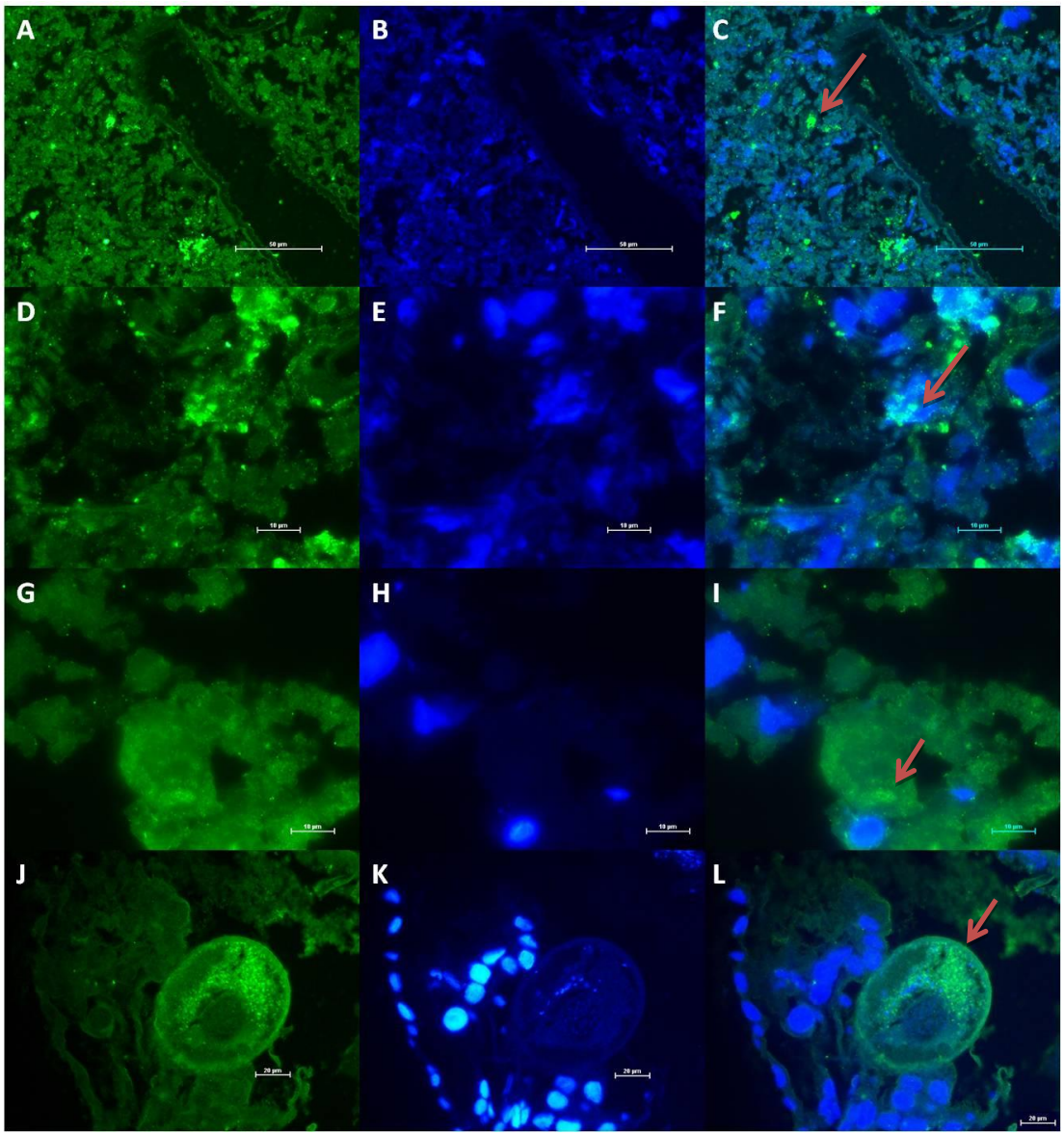

Figure 30: Immunofluorescence analysis of cattle tick tissues probed with anti-CRT antibodies.

Sections of 2-3 µm of different tick tissues were made and subsequently incubated with anti-CRT serum (1:100). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope. Nuclear DNA was stained with DAPI. (A) Salivary gland section under a fluorescein filter at 400x amplification; B) Salivary gland section with DAPI staining; (C) Merge of both staining in salivary gland; (D) Salivary gland section under a fluorescein filter at 1000x amplification; E) Salivary gland section with DAPI staining; (F) Merge of both staining in salivary gland; (G) Midgut section under a fluorescein filter at 1000x amplification; (H) Midgut section with DAPI staining; (I) Merge of both staining in midgut section; (J) Ovaries section under a fluorescein filter at 400x amplification; (K) Ovaries section with DAPI staining; (L) Merge of both staining in ovaries section. Bars, 50 µm (A, B, C); 10 µm (D-I) and 20 µm (G-H)

Regarding anti-TROSPA antibodies preliminary data shows that the native protein was recognized in the tissues analyzed (Figure 31). A clear membrane labeling is observed in salivary glands ducts (Figure 31 A-C) as well as in the ovaries section, where an immature egg is represented (Figure 31 G-I). The midgut section (Figure 31 D-F) also shows a disperse labeling suggesting that TROSPA protein is diffused in the wall of the tick intestine.

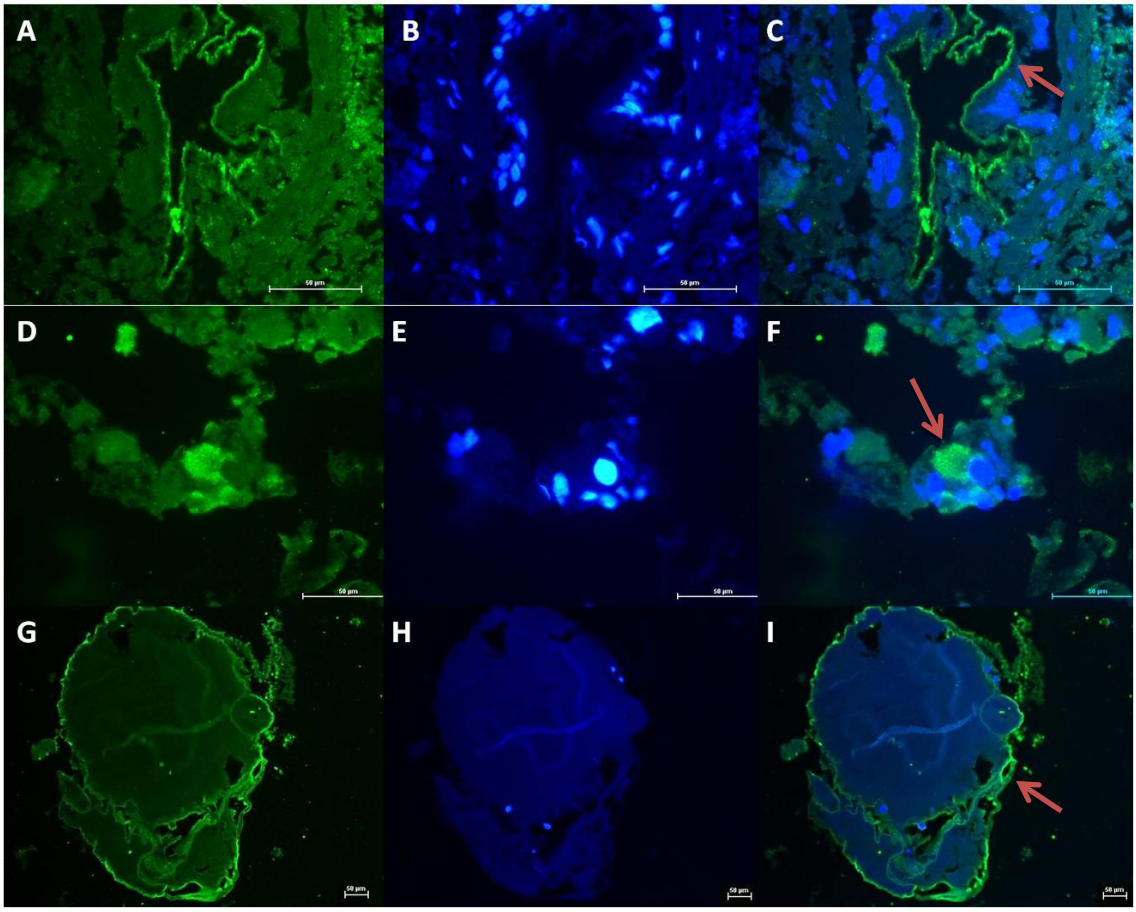

Figure 31: Immunofluorescence analysis of cattle tick tissues probed with anti- TROSPA antibodies.
Sections of 2-3 µm of different tick tissues were made and subsequently incubated with anti-TROSPA serum (1:100). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope. Nuclear DNA was stained with DAPI. (A) Salivary gland section under a fluorescein filter at 400X amplification; B) Salivary gland section with DAPI staining; (C) Merge of both staining in salivary gland; (D) Midgut section with under a fluorescein filter at 400X amplification; (E) Midgut section with DAPI staining; (F) Merge of both staining in midgut section; (G) Ovaries section under a fluorescein filter at 1000X amplification; (H) Ovaries section with DAPI staining, amplification 100X; (I) Merge of both staining in ovaries section. Bars 50 µm.

Negative control assays were conducted in order to validate results and determine tissues auto fluorescence (Figure 32 and Figure 40 in the appendix section).

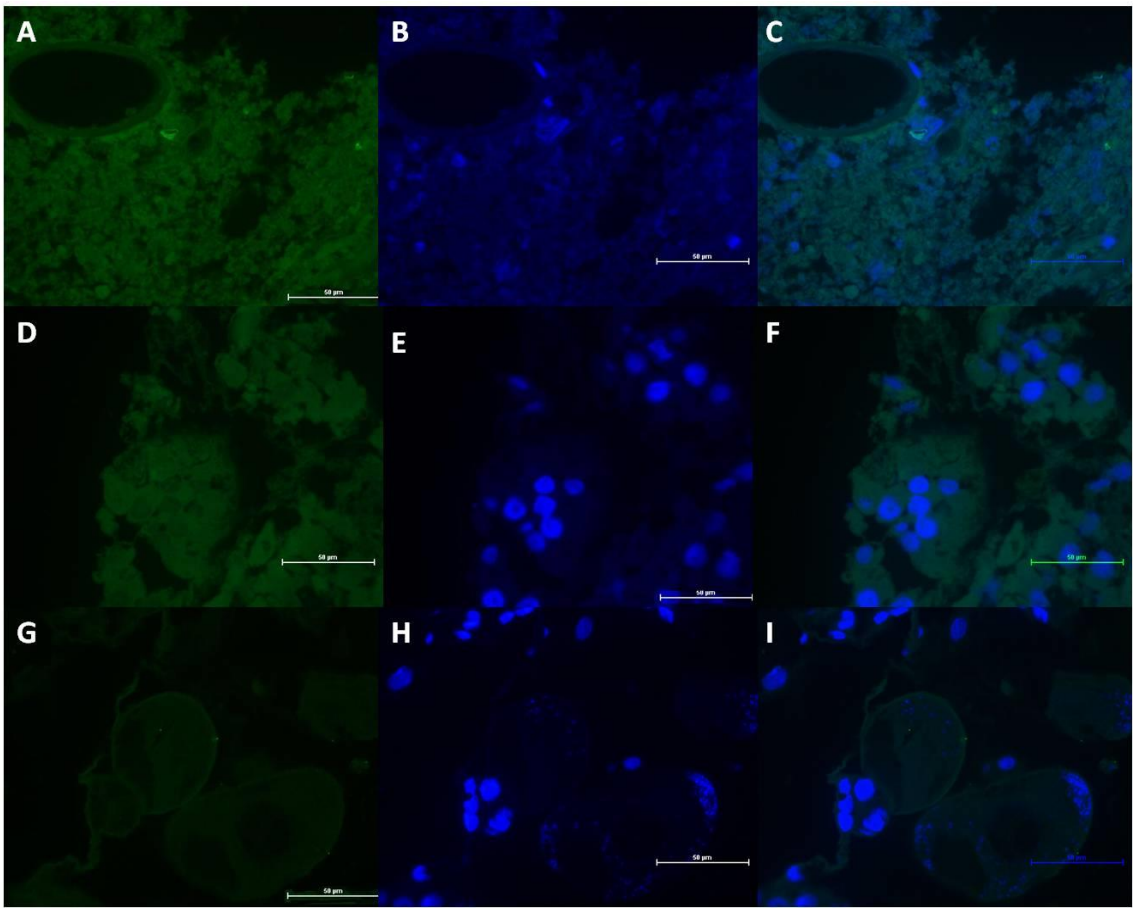

Figure 32: Immunofluorescence analysis of cattle tick tissues probed with pre-immune serum.
Sections of 2-3 µm of different tick tissues were made and subsequently incubated with pre-immune serum (1:100). Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope at 400x amplification. Nuclear DNA was stained with DAPI. (A) Salivary gland section under a fluorescein filter; B) Salivary gland section with DAPI staining; (C) Merge of both staining in salivary gland; (D) Midgut section with under a fluorescein filter; (E) Midgut section with DAPI staining; (F) Merge of both staining in midgut section; (G) Ovaries section under a fluorescein filter; H) Ovaries section with DAPI staining; (I) Merge of both staining in ovaries section. Bars 50 µm

To assess the differences of expression of TROSPA, a new immunofluorescence assay was carried on using anti-TROSPA antibodies against whole salivary glands of *B. bigemina*-infected *Boophilus* sp. tick and correspondent uninfected tissue (Figure 33). Despite weak, images show a positive signal in the infected tissue when compared to the uninfected one. These trials need to be optimized and are only showed here as preliminary data.

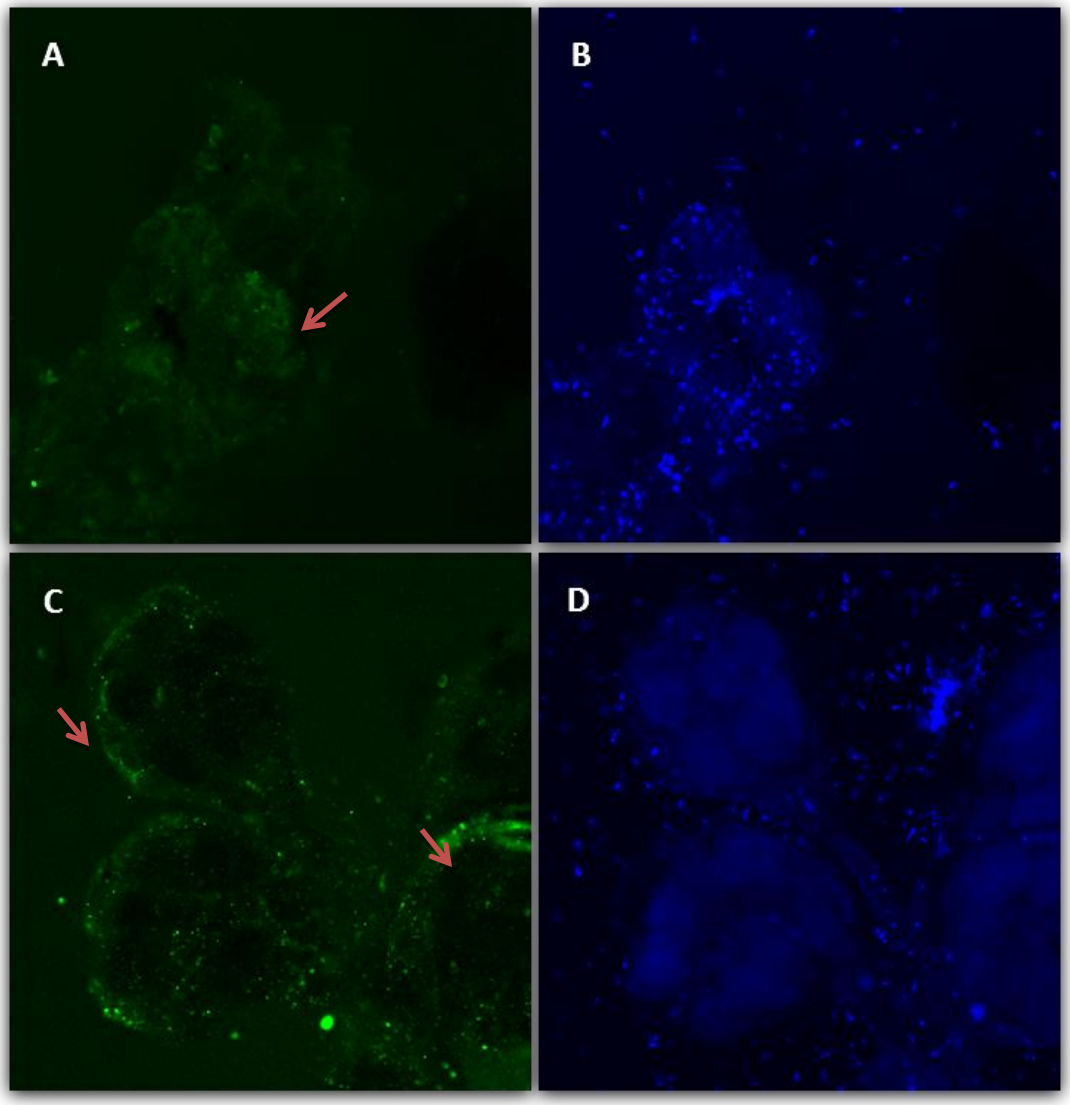

Figure 33: Immunofluorescence analysis of *R. annulatus* salivary glands.
Salivary glands were harvested and washed in PBS before fixed in 4% paraformaldehyde in poly-L-lysine treated slides and permeabilized using triton-X. Slides were blocked and subsequently incubated with anti-TROSPA serum. Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Zeiss LSM 710 confocal microscope 20× objective lens and presented as a single image for clarity. Nuclear DNA was stained with DAPI. A) Alexa Green staining in uninfected salivary glands. B) DAPI staining in non-infected salivary glands. C) Alexa Green staining in infected salivary glands. D) DAPI staining in infected salivary glands.

3.9 Capillary feeding assays

3.9.1. Effect of antibodies against tick proteins on tick weight, oviposition and pathogen infection.

Capillary feeding experiments (Figure 34) were conducted to evaluate the effect of antibodies against selected tick proteins on tick weight, oviposition and infection with *B. bigemina.*

Figure 34: Capillary feeding in *R. microplus* females.
Partially engorged females were manually recovered from calves and then cleaned, weighed and fixed on expandable polystyrene, with double face tape. Ticks were fed for 28 hours using capillary tubes as exemplified in the photo.

In the beginning of the assay, tick weight was registered showing a minimum 20mg and the maximum 60mg. In the total of 180 ticks only 5 failed to ingest blood. In those cases, as expected, there was weigh reduction because ticks naturally went on consuming energy for survival. In the present work, partially engorged *R. microplus* females fed artificially using capillaries had a significant increase in weight, from 86.8% to 195.2% (Table 11). After 28 h of artificial feeding, females presented a visible rounded idiosoma. Due to differences in the origin of antibodies the results are analyzed having in mind the proper negative control; pre-immune IgGs for groups feed with anti-proteins (TROSPA and SUB) IgGs and in the CRT case, the proper negative control was pre-immune serum.

Regarding tick weight, when ticks were fed with uninfected cattle blood supplemented with antibodies against tick proteins, only the group with anti-SUB IgGs showed a significant reduction in tick weight (24% reduction; P=0.0003) when compared to ticks fed on blood with pre-immune IgGs. When ticks were fed on *B. bigemina*-infected blood, groups with anti-TROSPA and anti-SUB IgGs showed reduction in tick weight of 18% (P=0.04) and 37% (P=0.001), respectively when compared to ticks fed on blood with pre-immune IgGs.

Table 11: Parameters associated to the feeding process of partially fed *Rhipicephalus (Boophilus) microplus* females by artificial feeding through capillaries, kept at 27°C and in relative humidity over 80%.

	Initial weight (mg) mean±SD	**Final weight (mg) mean±SD**	**Weight increment (mg) mean±SD**	**Weight increment % mean± SD**
Bovine blood	32,7±6,8	116,6±19,9	83,8±20,7	134,1±48,1
B.bigemina inf. bovine blood	32,9±9,4	126,2±41,6	93,3±36	195,2±45,6
Blood + preimnue IgGs	37,7±9,3	110,4±27,7	72,6±29,5	105,3±54,7
Blood + preimune serum	30,1±4,6	127,3±29,2	97,2±27,1	162,1±41,7
Blood + anti-CRT serum	29,7±2,7	110,9±20,8	81,2±21,7	139,1±47,8
Blood+ IgGs anti-TROSPA	32,8±9,3	123,1±23,3	90,3±28,9	153,3±67,6
Blood + IgGs anti SUB	31.6±11.1	86.9±26.3	55.3±20.2	93.9±38.7
Inf blood + preimune IgGs	32,9±6,4	107,4±34	81,7±19,2	126,9±43,5
Inf blood + preimune serum	30,3±8,3	91,4±29,2	61,1±26,1	104,5±43,3
Inf blood + anti CRT serum	36,7±7,6	125,7±37,6	89,1±33,6	122,8±42,6
Inf blood + IgGs anti TROSPA	37,5±10,5	104,6±24,3	67,1±22,8	95,5±40,9
Inf blood + IgGs anti SUB	32.7± 8.8	79.1±23.6	53.7±11.9	86.8±29.3

Fifteen female ticks per group were fed artificially using capillary tubes. Babesia bigemina infected bovine blood and healthy bovine blood alone or supplemented with antibodies was presented to ticks. After 28 hours ticks were weighted and held in a humidity chamber for 4 days to allow ticks to digest the blood meal.

After feeding, oviposition was reduced in ticks fed with anti-SUB antibodies, compared to ticks fed on blood supplemented with pre immune antibodies in infection and non-infection situation. A 70% reduction (P=0.001) was observed without the presence of *B .bigemina* in the blood meal and 43% reduction (P=0.01) in the presence of pathogen (Figure 35). No effect on oviposition was observed on the other groups.

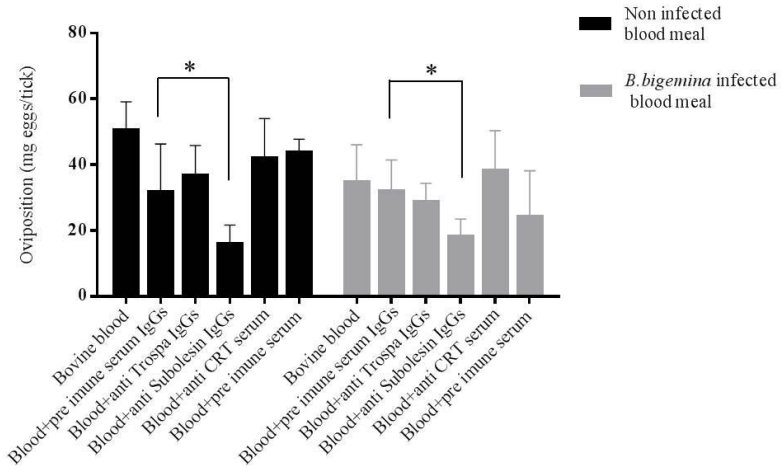

Figure 35: Effect of antibodies on tick oviposition.
Black bars: Ticks capillary fed on non-infected blood without and with pre immune serum/IgGs and anti-tick proteins serum/IgGs. Grey bars: Ticks capillary fed on *B. bigemina*-infected blood without and with pre immune serum/IgGs and anti-tick proteins serum/IgGs. Ticks (N=5) were incubated for oviposition after feeding, the egg mass weigh determined for each tick, expressed as Ave+S.D. (mg eggs/tick) and compared between the suitable group control and the other groups by Student´s t-test (*P<0.05).

Significant differences in pathogen infection levels were not found between groups fed on blood supplemented with pre immune IgGs/serum and anti-protein IgGs/serum (Figure 36).

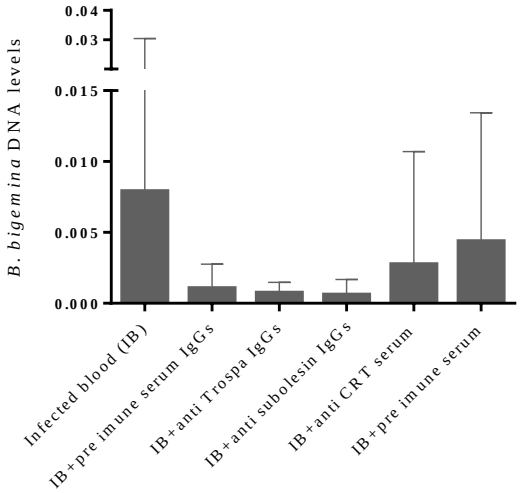

Figure 36: Effect of antibodies on *B. bigemina* infection.
B. bigemina DNA levels determined by PCR in ticks capillary-fed on *B. bigemina*-infected blood without and with preimmune and anti-tick proteins IgGs. The DNA levels were normalized against tick 16S rDNA, shown as Ave+S.D. Normalized Ct values (arbitrary units) and compared between the group with preimmune antibodies and the other groups by Student's t-Test (P>0.05; N=10).

3.9.2. Effect of antibodies and pathogen infection on the mRNA levels of genes encoding for tick proteins

mRNA levels were characterized in ticks fed on blood supplemented with pre immune IgGs/serum and anti-protein IgGs/serum. The ingestion of antibodies didn´t show a significant effect on the gene expression when compared to the control group in all proteins cases (P> 0.05) in either presence of infection or not. The mRNA levels ratio (fold change) between infected and uninfected ticks of the groups supplemented with antibodies was also analyzed (Figure 37). In all cases the ingestion of antibodies leads to a higher fold change.

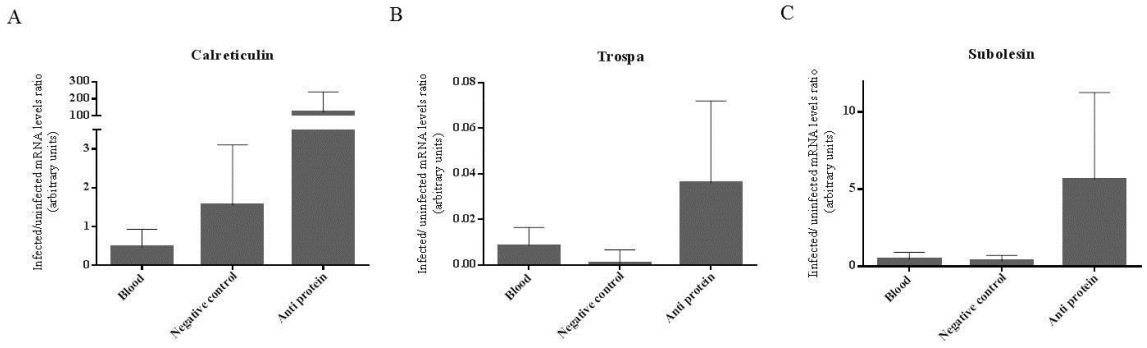

Figure 37: Fold change in expression of genes in the presence of *B. bigemina* infection.
The mRNA levels were normalized against tick 16S rRNA, shown as Ave+S.D. normalized Ct values (arbitrary units). (A) Effect of anti-CRT serum on *crt* gene expression. The *crt* mRNA levels were characterized by RT-PCR in ticks capillary-fed on uninfected blood alone or with the addition of preimmune serum and anti-CRT serum. (B) Effect of anti-trospa IgGs on *trospa* gene expression. The *trospa* mRNA levels were characterized by RT-PCR in ticks capillary-fed on uninfected blood alone or with the addition of pre immune IgGs and anti-TROSPA IgGs. (C) Effect of anti-SUB IgGs on *sub* gene expression. The *sub* mRNA levels were characterized by RT-PCR in ticks capillary-fed on uninfected blood alone or with the addition of pre immune IgGs and anti-sub IgGs.

Discussion

4.1. Antigens identification

In order to identify potential vaccine candidates, several studies have been performed focused on the characterization of the tick-pathogen interface at the molecular level (Macaluso et al., 2003, Mulenga et al., 2003, Nene et al., 2004, Rudenko et al., 2005, de la Fuente et al., 2007c, de la Fuente et al., 2007b, Villar et al., 2010, Zivkovic et al., 2010a, Mercado-Curiel et al., 2011, Heekin et al., 2012, Heekin et al., 2013). However, to our knowledge, this is the first report of differential expression of genes in an *R. annulatus* tick population infected with *B. bigemina.* In this work, we characterized *R. annulatus* genes differentially expressed in response to *B. bigemina* infection using SSH and real-time RT-PCR. Genes confirmed as differentially expressed in infected ticks were functionally characterized by RNAi approach to analyze their role during pathogen infection in the tick vector.

The SSH analysis used in this study to identify genes differentially expressed in *R annulatus* in response to *B. bigemina* infection did not result in a large variety of ESTs. These results are similar to those obtained for *A. marginale* using the same methodology and probably reflect tick-pathogen co-evolution (de la Fuente et al., 2007b, Villar et al., 2010, Zivkovic et al., 2010a, Mercado-Curiel et al., 2011). As expected, a large percentage of the identified ESTs did not show identity to known sequences with functional annotation. However, confirmed differentially expressed ESTs with predicted function suggested that these genes are involved in pathogen infection/multiplication and tick response to infection. As referred above, genes found to be differentially expressed after infection were further studied.

TROSPA. This protein was first described in *I. scapularis*, and recently in *I. persulcatus* (Konnai et al., 2012), as a receptor for *Borrelia burgdorferi* showing an enormous potential as a vaccine antigen to control bacterial infection in ticks (Pal et al., 2004, Hovius et al., 2007). Anti-TROSPA antibodies and gene knockdown reduced *B. burgdorferi* adherence to the *I. scapularis* gut *in vivo*, preventing efficient colonization of the vector and subsequently reducing pathogen transmission to the mammalian host (Pal et al., 2004). In *I. scapularis*, TROSPA mRNA levels increased following spirochete infection and decreased in response to tick engorgement, events that are temporally

linked to *B. burgdorferi* infection and transmission by the tick vector (Pal et al., 2004). Our results showed that this *R. annulatus* gene presents a high sequence identity to *Ixodes* spp. TROSPA was overexpressed in *B. bigemina*-infected ticks and played a similar role in both *R. annulatus* and *R. microplus* by leading to a significant lower infection after gene knockdown. These results suggested the possibility that *B. bigemina* uses a TROSPA ortholog receptor for infection of *Rhipicephalus* spp. tick cells encouraging research for the characterization of this molecule in *Babesia*-tick interactions and development of transmission blocking vaccines.

Calreticulin. CRT, a major endoplasmic reticulum calcium-binding protein, was overexpressed in *B. bigemina*-infected *R. annulatus.* This result is corroborated by a previous study where this protein showed to be up-regulated in ovaries of infected *R. microplus* ticks (Rachinsky et al., 2007). Gene knockdown, under the conditions undertaken here, reduced pathogen infection in *R. microplus* but not in *R. annulatus* ticks. The possible use of this protein in the development of protective immunity against parasites was suggested previously (Ferreira et al., 2002). Bovines immunized with a *R. microplus* recombinant calreticulin protein failed to produce a hyper immune serum against this molecule showing its low immunogenicity which could possibly be surpassed by the use of adjuvants and conjugation with highly immunogenic proteins (Ferreira et al., 2002). Calreticulin was found to be secreted in *Amblyomma* spp., *Dermacentor* spp. and *R. microplus* saliva suggesting a role for this protein during tick blood feeding (Jaworski et al., 1995, Ferreira et al., 2002), a result supported here, after gene knockdown in *R. annulatus* which resulted in reduced tick weight. These results suggested that calreticulin have a role during tick feeding and may be required for *Babesia* infection in some tick species.

Ricinusin. Ricinusin, longicin and microplusin are tick antimicrobial peptides included in the group of defensins, a well-conserved defense mechanism that is among the most important components of tick innate immunity (Tsuji et al., 2007, Silva et al., 2009, Hajdusek et al., 2013). Longicin has been shown to be a defensin against *B. gibsoni* infection in *Haemaphysalis longicornis* (Tsuji et al., 2007). Ricinusin was induced in *R. annulatus* in response to *B. bigemina* infection. Although ricinusin mRNA levels were

significantly higher in infected than in uninfected ticks, under the conditions of the present study, gene knockdown did not affect pathogen infection, thus suggesting that this molecule is not essential to control *B. bigemina* infection in *Boophilus* ticks.

Serum amyloid A. The expression of serum amyloid A increased in *R. annulatus* ticks infected with *B. bigemina* and gene knockdown resulted in lower infection levels in both *R. annulatus* and *R. microplus* without affecting tick weight after feeding. These results suggested that serum amyloid A may be part of tick response to the stress produced by *Babesia* infection but at the same time it was necessary for pathogen infection/multiplication in *Rhipicephalus* spp. ticks. Serum amyloid A is involved in host response to tissue injury and inflammation, which can increase their concentration over 1,000 fold and have various physiologic effects including antiplatelet activity (Urieli-Shoval et al., 2000). A serum amyloid putative protein was recently identified in the sialome of the soft tick *O. parkeri* (Francischetti et al., 2008) and in the *I. scapularis* genome (XM_002416454). However, a role for these proteins on tick pathogen infection and multiplication has not been previously described.

KTPI. The Kunitz-type protease inhibitors were the most represented ESTs in our dataset. Kunitz-type protease inhibitors are proteins of approximately 20 kDa with one or two disulfide bonds and a single reactive site (Major and Constabel, 2008). Some proteins belonging to the Kunitz family do not act as protease inhibitors or may have lectin-like carbohydrate-binding or invertase inhibitor activity (McCoy and Kortt, 1997, Macedo et al., 2004). Thus, determining a precise function of KTI-like proteins cannot be based only on primary sequence similarities, but requires *in vitro* assays for confirmation. Some Kunitz-type inhibitors are involved in tick defense mechanisms against pathogen infection, presumably via inhibition of microbial proteinases (Sasaki and Tanaka, 2008). In *Dermacentor variabilis*, tick cells invasion by *Rickettsia montanensis* is limited by a Kunitz-type protease inhibitor (Ceraul et al., 2011). Although Kunitz-type protease inhibitors were found to be up-regulated in other cases (Rachinsky et al., 2007) in the present study, in *B. bigemina*-infected ticks, KTPI mRNA levels were lower in infected *R. annulatus* than in uninfected ticks, probably suggesting a mechanism by which the pathogen manipulates gene expression to increase infection/multiplication. However,

KTPI knockdown did not affect *B. bigemina* infection in ticks and no other studies have been published on the effect of Kunitz-type protease inhibitors on *B. bigemina* infection. Interestingly, KTPI knockdown in *R. annulatus* and *R. microplus* reduced tick weight after feeding when compared to controls, suggesting a role for this protein during tick feeding.

Subolesin. This candidate tick protective antigen was initially discovered in *I. scapularis* and is conserved in many tick species. Subolesin play an important role in the immune response to pathogen infection through the control of genes involved in innate immunity (Almazan et al., 2003b, Goto et al., 2008, Galindo et al., 2009, Zivkovic et al., 2010b, de la Fuente et al., 2011). In previous experiments, it was showed that subolesin knockdown reduced *B. bigemina* infection in *R. microplus* (Merino et al., 2011b). However, herein subolesin knockdown did not affect *B. bigemina* infection in *R. annulatus*. The discrepancy between these results could be due to the fact that here adult ticks were injected with dsRNA before infestation, while in the previous experiment dsRNA was injected into replete females to infest cattle with resulting larvae (Merino et al., 2011b). As in previous experiments, subolesin knockdown reduced *R. annulatus* female tick weight after feeding (Almazan et al., 2010).

The effect of gene knockdown on pathogen infection could suggest genes necessary for pathogen infection/multiplication in the tick, and/or at least in some cases, genes affecting tick weight after RNAi may reduce the amount of blood ingested by ticks and thus the number of pathogens ingested during feeding.

RNAi was used in this study to analyze the effect of knockdown of selected genes on *B. bigemina* infection in ticks. Gene knockdown was carried out using *R. annulatus* sequences in both *R. annulatus* and *R. microplus* ticks due to the high similarity between their gene sequences. In *R. annulatus* but not in *R. microplus*, gene knockdown was successful for the all genes tested. Although dsRNA-mediated RNAi has been shown to function in *R. annulatus* using *R. microplus* sequences, the resulting phenotype was not similar between both tick species (Almazan et al., 2010). These results suggested that for some genes, sequence identity might not be sufficiently high for efficient gene

knockdown. The RNAi off-target effects (OTEs) (Scacheri et al., 2004) could not be ruled out in our gene knockdown experiments, as reported previously in *R. microplus* (Lew-Tabor et al., 2011). The absence of full tick genome data and the lack of a confirmed tick RNAi pathway, can under estimate the OTEs in current tick RNAi experiments (Lew-Tabor et al., 2011). Despite this, the use of long dsRNAs as gene knockdown treatments in ticks has been accepted as a routine method for validation/support of tick gene function (de la Fuente et al., 2007c, Smith et al., 2009, Merino et al., 2011a).

The present study identified new genes involved in the tick infection/multiplication of *B. bigemina*, improving our knowledge on the molecular mechanisms involved in tick-pathogen interactions. The results reported here increased our understanding on the role of tick genes in *Babesia bigemina* infection/multiplication, which is fundamental toward development of novel tick control measures. Some of the *R. annulatus* genes found in this study such as serum amyloid A, calreticulin and TROSPA could contribute to the development of novel vaccines designed to reduce tick infestations and prevent or minimize pathogen infection in ticks and transmission to vertebrate hosts.

4.2. Trospa and CRT expression and immunolocalization

Despite several attempts, it was not possible to obtain the nucleotide sequence of **TROSPA** using PCR having *I. scapularis* sequence as template; this difficulty was probably due to differences between these nucleotide sequences despite the high homology, as discussed in the previous section. In order to clarify the homology between proteins it will be necessary to obtain the full length *trospa* gene in subgenus *Boophilus* ticks and as the *R. microplus* genome is nearly complete, in a near future this issue will be once again addressed (Moolhuijzen et al., 2011, Bellgard et al., 2012).

The rTROSPA showed to be a good immunogenic antigen, since high titers were found after mice immunizations. Poly and monoclonal antibodies produced against the recombinant protein showed first to recognize the recombinant protein and second to recognize a native 27KDa in different tick tissues in both *R. annulatus* and *R. microplus.*

Pal et al. (2004) performed similar assays demonstrating that in *I. scapularis* anti-TROSPA serum recognized a native protein with ~55KDa. Moreover, the deduced TROSPA amino acid sequence showed several randomized repeats of up to 17 residues with an unusually high number of potential posttranslational modification signals, including O-glycosylation sites suggesting TROSPA undergoes glycosylation meaning that native TROSPA should be a glycoprotein (Pal et al., 2004). Glycosylation is one of the most abundant posttranslational modifications of proteins, and accumulating evidence indicates that the vast majority of proteins in eukaryotes are glycosylated (Roth et al., 2012). Nevertheless, treatment with variety of glycosidases revealed a failure in native TROSPA recognition so the idea of a ~55 KDa glycolated protein as to be regarded carefully and further investigations must be carried on. Within the present study, as referred, TROSPA anti-sera only recognized a single ~27kDa but there is no evidence that the recognized protein in cattle tick tissues might be a deglycosylated form of the native 55-kDa glycoprotein. This difference and the fact that native TROSPA was found not to be gut specific, contrary as it was demonstrated in *I. scapularis* (Pal et al., 2004) proposes that the role of this protein in sub genus *Boophilus* - *B. bigemina* interactions might be different in *Ixodes* sp. – *B. burgdorferi*. Obtaining the full length *trospa* gene in subgenus *Boophilus* ticks will for sure allow comparing sequences and enlighten this issue. Immunofluorescence assays proposes that native TROSPA in *R. microplus* ticks is present in tick salivary glands, midgut and ovaries again contrary to *I. scapularis* TROSPA (Pal et al., 2004) in where it is gut specific. This fact might be related to the *B. bigemina* infection process since after passing the midgut barrier pathogen disseminates similarly to other organs. Observations of IFA preparations points to this fact: while ovaries and salivary glands always showed the same localization pattern, in midgut, the signal was more disperse, what could be correlated to the timing of infection. Further investigations on the expression profile of TROSPA protein in the different tissues during infection will clarify this issue. The present preliminary IFA assays also demonstrates a clear membrane labeling pointing to a possible receptor role of this protein and to an increase of protein presence in infected salivary glands but, as already mentioned, more studies are necessary to clarify the involvement of this protein in pathogen infection process.

Once again, results showed that TROSPA remains a prominent candidate antigen for the control of pathogen infection.

Calreticulin showed to be up regulated in ticks infected with *B. bigemina* in SSH assays affecting tick infection when the expression of the protein was disturbed. This study reports the production of rCRT protein from *R. annulatus* as a potential antigen to induce anti-tick immunity. CRT is a calcium-binding protein divided into three domains: an N-terminal domain (N-domain), which is the most conserved domain among all CRTs (Michalak et al., 1999); an internal domain (P-domain), which binds Ca^{+2} with high affinity; and a C terminal domain (C-domain), which is highly acidic and exhibits a high capacity of Ca^{+2} binding (Baksh and Michalak, 1991). The deduced peptide sequence of *R. annulatus* CRT possesses all the three domains as well as the lack of an endoplasmic reticulum retention signal. Tick CRTs contain the KDEL sequence followed by the HEEL motif resulting in routing through a secretory pathway rather than being retained in the ER (Jaworski et al., 1995, Michalak et al., 1999, Kaewhom et al., 2008). Phylogenetic analysis show a high identity between tick CRTs and vertebrate CRTs; nevertheless, amino acid divergences among the full-length protein sequences of tick CRTs reveal a clear separation of tick CRTs (Parizi et al., 2009) indicating the existence of different immunogenic epitopes. In fact several studies points to the immunogenicity capacity of tick CRT (Jaworski et al., 1995, Xu et al., 2004, Gao et al., 2008, Parizi et al., 2009) and, recently, CRT was used as a biomarker on tick exposure (Alarcon-Chaidez et al., 2006, Vu Hai et al., 2013). Within the present study the rCRT showed to have a weak immunogenicity in mice in agreement with Ferreira et al (2002) that found bovines did not raise anti-tick CRT antibodies after vaccination with *R. microplus* rCRT. Differences in antigen production methodology, and on adjuvant used, together with natural genetic differences, may explain the discrepancies in results that have been obtained. As in Ferreira et al. study (2002), the rCRT used for immunization in the present study did not have the native conformation so some epitopes may not have been accessible limiting the immunological response of the host. Despite the low immunogenicity observed in mice, anti rCRT sera recognized the recombinant protein as well as the native protein, in cattle ticks. IFA assays revealed that, as expected, CRT is dispersed in salivary glands cells with no specific labeling (Jaworski et al., 1995, Gao et al., 2008, Kaewhom et al., 2008).

CRT was also found in ovaries and in this tissue a predominately a cytoplasmic localization is clear in immature eggs. Anti- CRT antibodies detected native CRT in tick midgut. Due to the many cellular functions, such as Ca^{2+} storage and signaling it was expected to find this protein in midgut (Michalak et al., 1999, Rachinsky et al., 2007).

Since the capacity of an antigen to induce cross-reactive immunity in the host is essential when considering candidate vaccines to control infestation by multiple tick species, the CRT protein may not be an ideal candidate due to the high similarity to host CRT; more studies using anti-CRT antibodies and different adjuvants will clarify their effect in vector-tick-pathogen interactions.

4.3. Characterization of tick proteins involved in tick-pathogen interactions as potential antigens for the control of tick infestation and pathogen infection.

One approach to increase the possibilities of identifying tick protective antigens is to combine RNAi functional studies with *in vitro* tick feeding. RNAi allows screening of a relatively large number of genes involved in tick-pathogen interactions while *in vitro* feeding with antibodies against selected candidate antigens can provide results resembling closer vaccine protective capacity. *In vitro* tick feeding is a technique that has been previously used for studies on tick biology and tick-pathogen interactions (Rau and Hannoun, 1968, Macaluso et al., 2001, Broadwater et al., 2002, Sonenshine et al., 2002, de la Fuente et al., 2005, Kocan et al., 2005, Kroeber and Guerin, 2007) and, more recently, to test the effect of antibodies added to blood meal on tick feeding.

Herein, *in vitro* capillary feeding assays were performed using polyclonal antibodies against the recombinant proteins TROSPA and CRT, selected due to its involvement in tick-*B. bigemina* interactions as demonstrated by RNAi functional studies. Antibodies anti-Subolesin were also used as control. The results showed the possibilities and limitations of this approach for the identification of candidate tick protective antigens.

Subolesin was again used in these experiments as a control due to the positive results obtained on previous vaccination trials performed to evaluate its applicability in

the control of *R. micropolus* infestations and *A. marginale* and *B. bigemina* infections (Merino et al., 2011b). As in previous experiments with vaccinated cattle (de la Fuente et al., 2011), anti-SUB antibodies reduced tick weight and oviposition on ticks fed on uninfected blood and also when ticks fed *B. bigemina*-infected blood suggesting ticks did ingest antibodies in a way resembling feeding on vaccinated cattle. *B. bigemina* DNA levels did not change in ticks fed on infected blood with anti-SUB antibodies, showing differences with the results of the vaccination trial in cattle (Merino et al., 2011b). The expression of *sub* has been shown to increase in response to *B. bigemina* infection in ticks (Merino et al., 2011b). However, in capillary fed ticks (without antibodies), *sub* mRNA levels did not increase in response to infection. Still, when comparing the mRNA levels ratio (fold change) between infected and uninfected ticks, the groups supplemented with anti-SUB antibodies showed an higher fold change suggesting an effect of the antibodies on the expression; low infection levels or even tick-to-tick variations, can confuse data on the effect of infection on the gene expression. To better understand the present results an analysis of a larger number of ticks and/or differences in pathogen infection between *in vivo* tick feeding and *in vitro* capillary feeding (Kocan et al., 2005) is required. SUB is involved in the control of various tick physiological processes including the immune response to pathogen infection and the expression of genes that are important for pathogen infection and multiplication and for tissue structure and function (Hajdusek et al., 2013, de la Fuente et al., 2006a). SUB is also involved in the regulation of its own expression (Naranjo et al., 2013). Therefore, the effect of anti-SUB antibodies on *sub* expression and possibly on SUB function could result in reduction of tick weigh and oviposition (Merino et al., 2011b). The ingestion of less infected blood and interference with pathogen infection and multiplication in ticks capillary-fed on blood with anti-SUB antibodies should have resulted in lower pathogen infection levels. However, this effect was not observed herein probably due to limitations of the *in vitro* capillary feeding system to study pathogen infection in ticks, at least for *B. bigemina* (Kocan et al., 2005).

TROSPA showed to be a good candidate for further characterization of their involvement in pathogen transmission as revealed by previous studies on gene knockdown by RNAi in which *B. bigemina* DNA levels were 83% and 70% lower in *R. annulatus* and *R. microplus*, respectively after gene silencing. Anti-TROSPA antibodies

reduced weight in ticks fed with infected blood suggesting TROSPA is related to pathogen transmission since in the absence of infection no effect is observed. Ticks fed with specific antibodies showed lower levels of pathogen DNA (when comparing to ticks fed with pre immune IgGs) but this results were not statistically significant. The limitations of the *in vitro* capillary feeding system to study tick-pathogen interactions previously discussed for SUB are probably the cause for these insufficient results. Once again, the fold change of TROSPA expression in infected ticks suggests that the specific antibodies had some effect in the regulation of gene expression. The blockage of TROSPA proteins by antibodies in infected tick tissues may, in some way, stimulate cells to express the correspondent *trospa* gene counterbalancing the effect of the antibodies or even the presence of the pathogen may modulate the expression of this gene in the tick. There is scarce information about *trospa* and all results encourage further studies on this gene.

Calreticulin is an attractive candidate to the development of an anti-tick vaccine since it is highly conserved between ticks and as it is secreted by the tick to the host integrating the group of exposed antigens not requiring re-vaccination: feeding of ticks would *per se* re-boost the host immune system and because of these arguments some hope is still deposit in CRT. Anti-CRT serum was used in capillary feeding assays. As in RNAi studies, in capillary fed ticks (without antibodies), *crt* mRNA levels increased in response to infection. No significant differences in weight or oviposition were found among groups fed with control serum and anti-CRT serum. Also pathogen DNA levels did not show a significant reduction when ticks were fed with anti-CRT antibodies. As referred, tick to tick variation may have masked the real effect of specific antibodies and probably a larger sample number would improve the discussion of the present results. Moreover, as CRT is a ubiquitous protein (Michalak et al., 1999) quantity of antibodies presented to ticks may have not been enough to promote visible effects. As in previous antigen cases, ingestion of antibodies lead to an increase in mRNA levels ratio (fold change) between infected and uninfected ticks, in groups supplemented with anti-CRT antibodies suggesting an effect of the antibodies on the expression. More studies have to be conducted to determine, for example, if using monoclonal antibodies directed to

specific epitopes (more immunogenic) would interfere with tick feeding and pathogen transmission.

In conclusion, the results reported here show that the use of *in vitro* tick capillary feeding has possibilities and limitations for the characterization of candidate tick protective antigens. Ticks fed by capillary feeding ingested antibodies added to the blood meal and the effect of these antibodies was shown on tick weight and oviposition. However, capillary feeding showed limitations in the study of pathogen infection in ticks associated with tick-to-tick variations in infection levels that require the analysis of a larger number of ticks and/or differences in pathogen infection processes between *in vivo* tick feeding and *in vitro* capillary feeding. Nevertheless, the combination of RNAi functional studies with tick capillary feeding using antibodies against selected proteins involved in tick-pathogen interactions should allow for a better and more efficient selection of candidate vaccine antigens. In the present study, the antigens, SUB and TROSPA, showed an effect on tick weight and/or oviposition in ticks fed with uninfected or infected blood containing antigen-specific IgGs. CRT antibodies did not show the expected influence putting this antigen aside as a candidate to vaccine development. These data together with previous results from RNAi studies suggest that SUB and TROSPA are good antigens candidates for vaccine development for the control of *R. microplus* infestations and *B. bigemina* infection. Further experiments, in particular *in vivo* vaccine trials, should be carried out to validate TROSPA.

4.4. General conclusions

The present study was designed to contribute to the development of a vaccine with impact in both tick and pathogen. The objective was the identification of genes differentially expressed in *Babesia bigemina* infected cattle ticks which was accomplished using suppression subtractive hybridization (SSH). Using this technique a relative small catalogue of genes up regulated in an infected tick population was obtained and after analysis of sequences, expression profile of some selected genes was investigated in *B. bigemina* infected and uninfected *Rhipicephalus annulatus* ticks by real time PCR. TROSPA, serum amyloid A, calreticulin, ricinusin and a kunitz type protease inhibitor were found to be significantly differentially expressed in infected cattle ticks.

The second part of this study is related to the validation of influence of the expression of these genes in pathogen transmission. RNA interference studies allow functional analyses of genes by disrupting their expression. With this approach it was established that the genes, calreticulin, TROSPA and serum amyloid A, present a significant influence on pathogen infection in cattle ticks. The final objective was the characterization of the identified antigens regarding the potential inclusion on a multi-recombinant antigen vaccine. To fulfill this intention two proteins were expressed, TROSPA and calreticulin, and antibodies against these antigens were produced in order to first, localize native proteins in tick tissues and second to evaluate their effect in the presence of infection during tick feeding. Immunolocalization studies showed that both anti TROSPA antibodies and anti calreticulin antibodies recognize the native antigen in tick ovaries, salivary glands and midgut. The following artificial capillary feeding assays showed that this methodology despite useful, has limitations: it cannot be used to substitute field *in vivo* vaccination or even used alone to validate antigens for vaccine development. Nevertheless, the combination of RNAi studies with artificial feeding technique used within the present work endorses the inclusion of TROSPA antigen in a field vaccination trial in cattle to assess about its protective effect in infection acquisition.

References

ABCAM. 2012. *Western Blotting - A begginer´s guide* [Online]. Available: http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf [Accessed 21-03-2013.

ABOULAILA, M., SIVAKUMAR, T., YOKOYAMA, N. & IGARASHI, I. 2010. Inhibitory effect of terpene nerolidol on the growth of *Babesia* parasites. *Parasitol Int,* 59**,** 278-82.

ALARCON-CHAIDEZ, F., RYAN, R., WIKEL, S., DARDICK, K., LAWLER, C., FOPPA, I. M., TOMAS, P., CUSHMAN, A., HSIEH, A., SPIELMAN, A., BOUCHARD, K. R., DIAS, F., ASLANZADEH, J. & KRAUSE, P. J. 2006. Confirmation of tick bite by detection of antibody to *Ixodes* calreticulin. *Clin Vaccine Immunol,* 13**,** 1217-22.

ALBERDI, M. P., DALBY, M. J., RODRIGUEZ-ANDRES, J., FAZAKERLEY, J. K., KOHL, A. & BELL-SAKYI, L. 2012. Detection and identification of putative bacterial endosymbionts and endogenous viruses in tick cell lines. *Ticks Tick Borne Dis,* 3**,** 137-146.

ALJAMALI, M. N., BIOR, A. D., SAUER, J. R. & ESSENBERG, R. C. 2003. RNA interference in ticks: a study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. *Insect Mol Biol,* 12**,** 299-305.

ALJAMALI, M. N., SAUER, J. R. & ESSENBERG, R. C. 2002. RNA interference: applicability in tick research. *Exp Appl Acarol,* 28**,** 89-96.

ALLEN, J. R. & HUMPHREYS, S. J. 1979. Immunization of Guinea-Pigs and cattle against ticks. *Nature,* 280**,** 491-493.

ALLSOPP, M., CAVALIERSMITH, T., DEWAAL, D. T. & ALLSOPP, B. A. 1994. Phylogeny and evolution of the piroplasms. *Parasitology,* 108**,** 147-152.

ALMAZAN, C., KOCAN, K. M., BERGMAN, D. K., GARCIA-GARCIA, J. C., BLOUIN, E. F. & DE LA FUENTE, J. 2003a. Characterization of genes transcribed in an *Ixodes scapularis* cell line that were idedntified by expression library immunization and analysis of expressed sequence tags. *Gene Ther Mol Biol,* 7**,** 43-59.

ALMAZAN, C., KOCAN, K. M., BERGMAN, D. K., GARCIA-GARCIA, J. C., BLOUIN, E. F. & DE LA FUENTE, J. 2003b. Identification of protective antigens for the control of *Ixodes scapularis* infestations using cDNA expression library immunization. *Vaccine,* 21**,** 1492-1501.

ALMAZAN, C., KOCAN, K. M., BLOUIN, E. E. & DE LA FUENTE, J. 2005. Vaccination with recombinant tick antigens for the control of *Ixodes scapularis* adult infestations. *Vaccine,* 23**,** 5294-5298.

ALMAZAN, C., LAGUNES, R., VILLAR, M., CANALES, M., ROSARIO-CRUZ, R., JONGEJAN, F. & DE LA FUENTE, J. 2010. Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations. *Parasitol Res,* 106**,** 471-479.

AMSDEN, J. R., WARMACK, S. & GUBBINS, P. O. 2005. Tick-borne bacterial, rickettsial, spirochetal, and protozoal infectious diseases in the United States: A comprehensive review. *Pharmacotherapy,* 25**,** 191-210.

ANTUNES, G. M. 2008. *Hemoparasitoses em bovinos de carne.* MSc Master Thesis, Universidade Técnica de Lisboa.

ARAGÃO, H. B. 1936. Ixodidas brasileiros e de alguns paizes limitrofes. *Mem Inst Oswaldo Cruz***,** 759-843.

ARISTOTLE. 350 B.C. *"The History of Animals." (Historia animalium)* [Online]. http://classics.mit.edu//Aristotle/history_anim.html. . [Accessed 22th July 2013 2013].

ARTHUR, D. R. 1965. Ticks in Egypt in 1500 B.C. *Nature,* 206**,** 1060-1061.

ASSADIAN, O. & STANEK, G. 2002. Theobald Smith - The discoverer of ticks as vectors of disease. *Wiener Klinische Wochenschrift,* 114**,** 479-481.

BABES, V. 1888. Sur l´hemoglobinurie bactérienne du boeuf. *Comptes Rendus de l'Academie des Sciences,* 107**,** 692–694.

BACCHI, C. J., NATHAN, H. C., HUTNER, S. H., DUCH, D. S. & NICHOL, C. A. 1981. Prevention by polyamines of the curative effect of amicarbalide and imidocarb for *Trypanosoma brucei* infections in mice. *Biochem Pharmacol,* 30**,** 883-6.

BAKSH, S. & MICHALAK, M. 1991. Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. *J Biol Chem,* 266**,** 21458-65.

BARKER, S. C. & MURRELL, A. 2004. Systematics and evolution of ticks with a list of valid genus and species names. *Parasitology,* 129**,** S15-S36.

BARRY, G., ALBERDI, P., SCHNETTLER, E., WEISHEIT, S., KOHL, A., FAZAKERLEY, J. K. & BELL-SAKYI, L. 2013. Gene silencing in tick cell lines using small interfering or long double-stranded RNA. *Exp Appl Acarol,* 59**,** 319-338.

BARRY, M. A., LAI, W. C. & JOHNSTON, S. A. 1995. Protection against mycoplasma infection using expression-library immunization. *Nature,* 377**,** 632-635.

BEATI, L & KEIRANS, J.E. 2001. Analysis of the systematic relationships among ticks of the genera Rhipicephalus and Boophilus (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. *J Parasitol*. 87(1):32-48.

BELLGARD, M. I., MOOLHUIJZEN, P. M., GUERRERO, F. D., SCHIBECI, D., RODRIGUEZ-VALLE, M., PETERSON, D. G., DOWD, S. E., BARRERO, R., HUNTER, A., MILLER, R. J. & LEW-TABOR, A. E. 2012. CattleTickBase: an integrated Internet-based bioinformatics resource for. *Int J Parasitol,* 42**,** 161-9.

BENAVIDES, M. V. & SACCO, A. M. 2007. Differential *Bos taurus* cattle response to *Babesia bovis* infection. *Vet Parasitol,* 150**,** 54-64.

BENSACI, M., BHATTACHARYA, D., CLARK, R. & HU, L. T. 2012. Oral vaccination with vaccinia virus expressing the tick antigen subolesin inhibits tick feeding and transmission of *Borrelia burgdorferi*. *Vaccine,* 30**,** 6040-6.

BERNARD, P., GABANT, P., BAHASSI, E. M. & COUTURIER, M. 1994. Positive selection vectors using F-plasmid CCDB killer gene. *Gene,* 148**,** 71-74.

BLOUIN, E. E., MANZANO-ROMAN, R., DE LA FUENTE, J. & KOCAN, K. M. 2008. Defining the role of Subolesin in tick cell culture by use of RNA interference. *Animal Biodiversity and Emerging Diseases: Prediction and Prevention,* 1149**,** 41-44.

BOCK, R., JACKSON, L., DE VOS, A. & JORGENSEN, W. 2004. Babesiosis of cattle. *Parasitology,* 129**,** S247-S269.

BOCK, R. E., DEVOS, A. J., KINGSTON, T. G. & MCLELLAN, D. J. 1997. Effect of breed of cattle on innate resistance to infection with *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*. *Aus Vet J,* 75**,** 337-340.

BOLDBAATAR, D., BATTSETSEG, B., MATSUO, T., HATTA, T., UMEMIYA-SHIRAFUJI, R., XUAN, X. & FUJISAKI, K. 2008. Tick vitellogenin receptor reveals critical role in oocyte development and transovarial transmission of Babesia parasite. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire,* 86**,** 331-344.

BORK, S., YOKOYAMA, N., MATSUO, T., CLAVERIA, F. G., FUJISAKI, K. & IGARASHI, I. 2003. Growth inhibitory effect of triclosan on equine and bovine Babesia parasites. *Am J Trop Med Hyg,* 68**,** 334-40.

BOSE, R., JORGENSEN, W. K., DALGLIESH, R. J., FRIEDHOFF, K. T. & DE VOS, A. J. 1995. Current state and future trends in the diagnosis of Babesiosis. *Vet Parasitol,* 57**,** 61-74.

BRACK, C., DELAIN, E., RIOU, G. & FESTY, B. 1972. Molecular organization of the kinetoplast DNA of *Trypanosoma cruzi* treated with berenil, a DNA interacting drug. *J Ultrastruct Res,* 39**,** 568-79.

BRIANTI, E., OTRANTO, D., DANTAS-TORRES, F., WEIGL, S., LATROFA, M. S., GAGLIO, G., NAPOLI, E., BRUCATO, G., CAUQUIL, L., GIANNETTO, S. & BAIN, O. 2012. *Rhipicephalus sanguineus* (Ixodida, Ixodidae) as intermediate host of a canine neglected filarial species with dermal microfilariae. *Vet Parasitol,* 183**,** 330-337.

BROADWATER, A. H., SONENSHINE, D. E., HYNES, W. L., CERAUL, S. & DE SILVA, A. M. 2002. Glass capillary tube feeding: A method for infecting nymphal *Ixodes scapularis* (Acari : Ixodidae) with the Lyme disease spirochete *Borrelia burgdorferi*. *J Med Entomol,* 39**,** 285-292.

BROWN, W. C., NORIMINE, J., GOFF, W. L., SUAREZ, C. E. & MCELWAIN, T. F. 2006a. Prospects for recombinant vaccines against *Babesia bovis* and related parasites. *Parasite Immunology,* 28**,** 315-327.

BROWN, W. C., NORIMINE, J., KNOWLES, D. P. & GOFF, W. L. 2006b. Immune control of *Babesia bovis* infection. *Vet Parasitol,* 138**,** 75-87.

BROWN, W. C. & PALMER, G. H. 1999. Designing blood-stage vaccines against *Babesia bovis* and *B. bigemina*. *Parasitol Today,* 15**,** 275-281.

BULING, A., CRIADO-FORNELIO, A., ASENZO, G., BENITEZ, D., BARBA-CARRETERO, J. C. & FLORIN-CHRISTENSEN, M. 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. *Vet Parasitol,* 147**,** 16-25.

CAEIRO, V. 1999. General review of tick species present in Portugal. *Parassitologia,* 41 Suppl 1**,** 11-5.

CALLOW, L. L. & HOYTE, H. M. D. 1961a. The separation of *Babesia bigemina* from *Babesia argentina* and *Theileria mutans*. *Aus Vet J,* 37**,** 66-70.

CALLOW, L. L. & HOYTE, H. M. D. 1961b. Transmission experiments using *Babesia bigemina, Theileria mutans* , *Borrelia* sp. and the tick cattle *Boophilus microplus*. *Aus Vet J,* 37**,** 381-390.

CAPERUCCI, D., BECHARA, G. H. & CAMARGO MATHIAS, M. I. 2010. Ultrastructure features of the midgut of the female adult Amblyomma cajennense ticks Fabricius, 1787 (Acari: Ixodidae) in several feeding stages and subjected to three infestations. *Micron,* 41**,** 710-21.

CEN-AGUILAR, J. F., RODRIGUEZ-VIVAS, R. I., DOMINGUEZ-ALPIZAR, J. L. & WAGNER, G. G. 1998. Studies on the effect of infection by *Babesia* sp. on oviposition of *Boophilus microplus* engorged females naturally infected in the Mexican tropics. *Vet Parasitol,* 78**,** 253-257.

CERAUL, S. M., CHUNG, A., SEARS, K. T., POPOV, V. L., BEIER-SEXTON, M., RAHMAN, M. S. & AZAD, A. F. 2011. A Kunitz protease inhibitor from *Dermacentor variabilis*, a vector for spotted fever group rickettsiae, limits *Rickettsia montanensis* invasion. *Infect Immun,* 79**,** 321-9.

CHAUVIN, A., MOREAU, E., BONNET, S., PLANTARD, O. & MALANDRIN, L. 2009. *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet Res,* 40 (2), 37

COLDHAM, N. G., MOORE, A. S., SIVAPATHASUNDARAM, S. & SAUER, M. J. 1994. Imidocarb depletion from cattle liver and mechanism of retention in isolated bovine hepatocytes. *Analyst,* 119**,** 2549-52.

COLWELL, D. D., DANTAS-TORRES, F. & OTRANTO, D. 2011. Vector-borne parasitic zoonoses: Emerging scenarios and new perspectives. *Vet Parasitol,* 182**,** 14-21.

COONS, L. B. & ALBERTI, G. 1999. The acari-ticks. *In:* HARRISON, F. W. & FOELIX, R. (eds.) *Microscopic anatomy of invertebrates. Chelicerate Arthropoda.* New York: Wiley.

CRIADO-FORNELIO, A., BULING, A., ASENZO, G., BENITEZ, D., FLORIN-CHRISTENSEN, M., GONZALEZ-OLIVA, A., HENRIQUES, G., SILVA, M., ALONGI, A., AGNONE, A., TORINA, A. & MADRUGA, C. R. 2009. Development of fluorogenic probe-based PCR assays for the detection and quantification of bovine piroplasmids. *Vet Parasitol,* 162**,** 200-206.

DANTAS-TORRES, F., CHOMEL, B. B. & OTRANTO, D. 2012. Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol,* 28**,** 437-446.

DAVIES, C. R., JONES, L. D. & NUTTALL, P. A. 1986. Experimental studies on the transmission cycle of Thogoto virus, a candidate orthomyxovirus, in *Rhipicephalus appendiculatus*. *Am J Trol Medicine Hyg,* 35**,** 1256-1262.

DE LA FUENTE, J., ALMAZAN, C., BLAS-MACHADO, U., NARANJO, V., MANGOLD, A. J., BLOUIN, E. F., GORTAZAR, C. & KOCAN, K. M. 2006a. The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood ingestion and reproduction. *Vaccine,* 24**,** 4082-4095.

DE LA FUENTE, J., ALMAZAN, C., BLOUIN, E. F., NARANJO, V. & KOCAN, K. M. 2006b. Reduction of tick infections with *Anaplasma marginale* and *A. phagocytophilum* by targeting the tick protective antigen subolesin. *Parasitol Res,* 100**,** 85-91.

DE LA FUENTE, J., ALMAZAN, C., CANALES, M., PEREZ DE LA LASTRA, J. M., KOCAN, K. M. & WILLADSEN, P. 2007a. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Animal health research reviews / Conference of Research Workers in Animal Diseases,* 8**,** 23-8.

DE LA FUENTE, J., ALMAZAN, C., NARANJO, V., BLOUIN, E. F., MEYER, J. M. & KOCAN, K. M. 2006c. Autocidal control of ticks by silencing of a single gene by RNA interference. *Biochem Biophys Res Commun,* 344**,** 332-338.

DE LA FUENTE, J., ALMAZAN, C., VAN DEN BUSSCHE, R. A., BOWMAN, J., YOSHIOKA, J. H. & KOCAN, K. M. 2005. Characterization of genetic diversity in *Dermacentor andersoni* (Acari : Ixodidae) with body size and weight polymorphism. *Exp Parasitol,* 109**,** 16-26.

DE LA FUENTE, J., BLOUIN, E. F., MANZANO-ROMAN, R., NARANJO, V., ALMAZAN, C., PEREZ DE LA LASTRA, J. M., ZIVKOVIC, Z., JONGEJAN, F. & KOCAN, K. M. 2007b. Functional genomic studies of tick cells in response to infection with the cattle pathogen, *Anaplasma marginale*. *Genomics,* 90**,** 712-22.

DE LA FUENTE, J., ESTRADA-PENA, A., VENZAL, J. M., KOCAN, K. M. & SONENSHINE, D. E. 2008a. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci,* 13**,** 6938-6946.

DE LA FUENTE, J. & KOCAN, K. M. 2003. Advances in the identification and characterization of protective antigens for recombinant vaccines against tick infestations. *Expert rev vaccines,* 2**,** 583-93.

DE LA FUENTE, J. & KOCAN, K. M. 2006. Strategies for development of vaccines for control of ixodid tick species. *Parasite Immunol,* 28**,** 275-283.

DE LA FUENTE, J., KOCAN, K. M., ALMAZAN, C. & BLOUIN, E. F. 2007c. RNA interference for the study and genetic manipulation of ticks. *Trends Parasitol,* 23**,** 427-433.

DE LA FUENTE, J., MARITZ-OLIVIER, C., NARANJO, V., AYOUBI, P., NIJHOF, A. M., ALMAZAN, C., CANALES, M., PEREZ DE LA LASTRA, J. M., GALINDO, R. C., BLOUIN, E. F., GORTAZAR, C., JONGEJAN, F. & KOCAN, K. M. 2008b. Evidence of the role of tick subolesin in gene expression. *BMC Genomics,* 9**,** 372.

DE LA FUENTE, J., MORENO-CID, J. A., CANALES, M., VILLAR, M., PEREZ DE LA LASTRA, J. M., KOCAN, K. M., GALINDO, R. C., ALMAZAN, C. & BLOUIN, E. F. 2011. Targeting arthropod subolesin/akirin for the development of

a universal vaccine for control of vector infestations and pathogen transmission. *Vet Parasitol,* 181**,** 17-22.

DE LA FUENTE, J., RODRIGUEZ, M. & GARCIA-GARCIA, J. C. 2000. Immunological control of ticks through vaccination with *Boophilus microplus* gut antigens. *Ann N Y Acad Sci,* 916**,** 617-21.

DE LA FUENTE, J., RODRIGUEZ, M., REDONDO, M., MONTERO, C., GARCIA-GARCIA, J. C., MENDEZ, L., SERRANO, E., VALDES, M., ENRIQUEZ, A., CANALES, M., RAMOS, E., BOUE, O., MACHADO, H., LLEONART, R., DE ARMAS, C. A., REY, S., RODRIGUEZ, J. L., ARTILES, M. & GARCIA, L. 1998. Field studies and cost-effectiveness analysis of vaccination with Gavac (TM) against the cattle tick *Boophilus microplus*. *Vaccine,* 16**,** 366-373.

DE LEON, A. A. P., STRICKMAN, D. A., KNOWLES, D. P., FISH, D., THACKER, E., DE LA FUENTE, J., KRAUSE, P. J., WIKEL, S. K., MILLER, R. S., WAGNER, G. G., ALMAZAN, C., HILLMAN, R., MESSENGER, M. T., UGSTAD, P. O., DUHAIME, R. A., TEEL, P. D., ORTEGA-SANTOS, A., HEWITT, D. G., BOWERS, E. J., BENT, S. J., COCHRAN, M. H., MCELWAIN, T. F., SCOLES, G. A., SUAREZ, C. E., DAVEY, R., FREEMAN, J. M. H., LOHMEYER, K., LI, A. Y., GUERRERO, F. D., KAMMLAH, D. M., PHILLIPS, P., POUND, J. M., GRP EMERGING, B. & ONE, H. 2010. One Health approach to identify research needs in bovine and human babesioses: workshop report. *Parasites & Vectors,* 3.

DE VOS, A. J. 1979. Epidemiology and control of bovine babesiosis in South Africa. *J S Afr Vet Assoc,* 50**,** 357-62.

DE VOS, S., ZEINSTRA, L., TAOUFIK, O., WILLADSEN, P. & JONGEJAN, F. 2001. Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. *Exp Appl Acarol,* 25**,** 245-61.

DE WAAL, D. T. & COMBRINK, M. P. 2006. Live vaccines against bovine babesiosis. *Vet Parasitol,* 138**,** 88-96.

DIATCHENKO, L., CHENCHIK, A., SIEBERT, P. & LUKIANOV, S. 1995. Suppression subtractive hybridization technique. *Faseb Journal,* 9**,** A1421-A1421.

DIATCHENKO, L., LUKYANOV, S., LAU, Y. F. C. & SIEBERT, P. D. 1999. Suppression subtractive hybridization: A versatile method for identifying differentially expressed genes. *Cdna Preparation and Characterization,* 303**,** 349-380.

DILCHER, M., ALVES, M. J., FINKEISEN, D., HUFERT, F. & WEIDMANN, M. 2012. Genetic characterization of Bhanja virus and Palma virus, two tick-borne phleboviruses. *Virus Genes,* 45**,** 311-315.

DOMINGOS, A., ANTUNES, S., BORGES, L. & ROSARIO, V. E. 2013. Approaches towards tick and tick-borne diseases control. *Rev Soc Bras Med Trop,* 46.

DUMPIS, U., CROOK, D. & OKSI, J. 1999. Tick-borne encephalitis. *Clin Infect Dis,* 28**,** 882-890.

ECKER, J. R. & DAVIS, R. W. 1986. Inhibition of gene expression in plant cells by expression of antisense RNA. *P Natl Acad Sci USA,* 83**,** 5372-5376.

ELSHOURA, S. M. 1990. Nuttalliella namaqua (Acarina, Ixodoidea, Nuttalliellidae) – Redescription of the female morphology in relation to the families Argasidae and ixodidae- review article. *Acarologia,* 31**,** 349-355.

EMBERS, M. E. & NARASIMHAN, S. 2013. Vaccination against Lyme disease: past, present, and future. *Front Cell Infect Microbiol,* 3**,** 6.

ESTRADA-PENA, A., GRAY, J. S., KAHL, O., LANE, R. S. & NIJHOF, A. M. 2013. Research on the ecology of ticks and tick-borne pathogens-methodological principles and caveats. *Front Cell Infect Microbiol,* 3.

ESTRADA-PENA, A. & JONGEJAN, F. 1999. Ticks feeding on humans: a review of records on human-biting Ixodoidea with special reference to pathogen transmission. *Exp Appl Acarol,* 23**,** 685-715.

ESTRADA-PENA, A. & SANTOS-SILVA, M. M. 2005. The distribution of ticks (Acari:Ixodidae) of domestic livestock in Portugal. *Exp Appl Acarol,* 36**,** 233-246.

FERREIRA, C. A. S., VAZ, I. D., DA SILVA, S. S., HAAG, K. L., VALENZUELA, J. G. & MASUDA, A. 2002. Cloning and partial characterization of a *Boophilus microplus* (Acari : Ixodidae) calreticulin. *Exp Parasitol,* 101**,** 25-34.

FIGUEROA, J. V., CHIEVES, L. P., JOHNSON, G. S. & BUENING, G. M. 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina, B. bovis* and *Anaplasma marginale* DNA in bovine blood. *Vet Parasitol,* 50**,** 69-81.

FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature,* 391**,** 806-11.

FISH, L., LEIBOVICH, B., KRIGEL, Y., MCELWAIN, T. & SHKAP, V. 2008. Vaccination of cattle against *B. bovis* infection with live attenuated parasites and non-viable immunogens. *Vaccine,* 26 Suppl 6**,** G29-33.

FJOSE, A., ELLINGSEN, S., WARGELIUS, A. & SEO, H. C. 2001. RNA interference: mechanisms and applications. *Biotechnology annual review,* 7**,** 31-57.

FLORIN-CHRISTENSEN, M. & SCHNITTGER, L. 2009. Piroplasmids and ticks: a long-lasting intimate relationship. *Front Biosci,* 14**,** 3064-3073.

FRANCISCHETTI, I. M., MANS, B. J., MENG, Z., GUDDERRA, N., VEENSTRA, T. D., PHAM, V. M. & RIBEIRO, J. M. 2008. An insight into the sialome of the soft tick, *Ornithodorus parkeri*. *Insect Biochem Mol Biol,* 38**,** 1-21.

FRISCH, J. E. 1999. Towards a permanent solution for controlling cattle ticks. *Int J Parasitol,* 29**,** 57-71; discussion 73-5.

GALINDO, R. C., DONCEL-PEREZ, E., ZIVKOVIC, Z., NARANJO, V., GORTAZAR, C., MANGOLD, A. J., MARTIN-HERNANDO, M. P., KOCAN, K. M. & DE LA FUENTE, J. 2009. Tick subolesin is an ortholog of the akirins described in insects and vertebrates. *Dev Comp Immunol,* 33**,** 612-7.

GAO, J., LUO, J., FAN, R., FINGERLE, V., GUAN, G., LIU, Z., LI, Y., ZHAO, H., MA, M., LIU, J., LIU, A., REN, Q., DANG, Z., SUGIMOTO, C. & YIN, H. 2008. Cloning and characterization of a cDNA clone encoding calreticulin from *Haemaphysalis qinghaiensis* (Acari : Ixodidae). *Parasitology Research,* 102**,** 737-746.

GARCIA, S., BILLECOCQ, A., CRANCE, J. M., MUNDERLOH, U., GARIN, D. & BOULOY, M. 2005. Nairovirus RNA sequences expressed by a Semliki Forest virus replicon induce RNA interference in tick cells. *Journal of Virology,* 79**,** 8942-8947.

GARNHAM, P. C. C., DONNELLY, J., HOOGSTRA.H, KENNEDY, C. C. & WALTON, G. A. 1969. Human babesiosis in Ireland – Further observations and medical significance of this infection. *British Medical Journal,* 4**,** 768-&.

GEORGE, J. E., POUND, J. M. & DAVEY, R. B. 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology,* 129**,** S353-S366.

GHOSH, S., AZHAHIANAMBI, P. & YADAV, M. P. 2007. Upcoming and future strategies of tick control: a review. *Journal of vector borne diseases,* 44**,** 79-89.

GOFF, W. L., JOHNSON, W. C., MOLLOY, J. B., JORGENSEN, W. K., WALDRON, S. J., FIGUEROA, J. V., MATTHEE, O., ADAMS, D. S., MCGUIRE, T. C., PINO, I., MOSQUEDA, J., PALMER, G. H., SUAREZ, C. E., KNOWLES, D. P. & MCELWAIN, T. F. 2008. Validation of a competitive enzyme-linked immunosorbent assay for detection of *Babesia bigemina* antibodies in cattle. *Clin Vaccine Immunol,* 15**,** 1316-21.

GOHIL, S., HERRMANN, S., GUNTHER, S. & COOKE, B. M. 2013. Bovine babesiosis in the 21st century: Advances in biology and functional genomics. *Int J Parasitol,* 43**,** 125-132.

GOMES, J., SOARES, R., SANTOS, M., SANTOS-GOMES, G., BOTELHO, A., AMARO, A. & INACIO, J. 2013. Detection of *Theileria* and *Babesia* infections amongst asymptomatic cattle in Portugal. *Ticks Tick Borne Dis,* 4**,** 148-51.

GOO, Y. K., TERKAWI, M. A., JIA, H., ABOGE, G. O., OOKA, H., NELSON, B., KIM, S., SUNAGA, F., NAMIKAWA, K., IGARASHI, I., NISHIKAWA, Y. & XUAN, X. 2010. Artesunate, a potential drug for treatment of *Babesia* infection. *Parasitol Int,* 59**,** 481-6.

GOTO, A., MATSUSHITA, K., GESELLCHEN, V., EL CHAMY, L., KUTTENKEULER, D., TAKEUCHI, O., HOFFMANN, J. A., AKIRA, S., BOUTROS, M. & REICHHART, J.-M. 2008. Akirins are highly conserved

nuclear proteins required for NF-kappa B-dependent gene expression in drosophila and mice. *Nature Immunology,* 9**,** 97-104.

GRAF, J. F., GOGOLEWSKI, R., LEACH-BING, N., SABATINI, G. A., MOLENTO, M. B., BORDIN, E. L. & ARANTES, G. J. 2004. Tick control: an industry point of view. *Parasitol,* 129**,** S427-S442.

GRAY, J. S. 1982. The effects of the piroplasm *Babesia bigemina* on the survival and reproduction of the blue tick *Boophilus decoloratus*. *Journal of Invertebrate Pathology,* 39**,** 413-415.

GUBBELS, J. M., DE VOS, A. P., VAN DER WEIDE, M., VISERAS, J., SCHOULS, L. M., DE VRIES, E. & JONGEJAN, F. 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *Journal of Clinical Microbiology,* 37**,** 1782-1789.

GUERRERO, F. D., BENDELE, K. G., DAVEY, R. B. & GEORGE, J. E. 2007. Detection of *Babesia bigemina* infection in strains of *Rhipicephalus (Boophilus) microplus* collected from outbreaks in South Texas. *Vet Parasitol,* 145**,** 156-163.

GUERRERO, F. D., LOVIS, L. & MARTINS, J. R. 2012a. Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. *Rev Bras Parasitol Vet,* 21**,** 1-6.

GUERRERO, F. D., MILLER, R. J. & DE LEON, A. A. P. 2012b. Cattle tick vaccines: Many candidate antigens, but will a commercially viable product emerge? *Int J Parasitol,* 42**,** 421-427.

GUO, Y. J., RIBEIRO, J. M. C., ANDERSON, J. M. & BOUR, S. 2009. dCAS: a desktop application for cDNA sequence annotation. *Bioinformatics,* 25**,** 1195-1196.

HAJDUSEK, O., ALMAZAN, C., LOOSOVA, G., VILLAR, M., CANALES, M., GRUBHOFFER, L., KOPACEK, P. & DE LA FUENTE, J. 2010. Characterization of ferritin 2 for the control of tick infestations. *Vaccine,* 28**,** 2993-2998.

HAJDUSEK, O., SIMA, R., AYLLON, N., JALOVECKA, M., PERNER, J., DE LA FUENTE, J. & KOPACEK, P. 2013. Interaction of the tick immune system with transmitted pathogens. *Frontiers in cellular and infection microbiology,* 3**,** 26-26.

HAJDUSEK, O., SOJKA, D., KOPACEK, P., BURESOVA, V., FRANTA, Z., SAUMAN, I., WINZERLING, J. & GRUBHOFFER, L. 2009. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. *P Natl Acad Sci USA,* 106**,** 1033-1038.

HAVLIKOVA, S., ROLLER, L., KOCI, J., TRIMNELL, A. R., KAZIMIROVA, M., KLEMPA, B. & NUTTALL, P. A. 2009. Functional role of 64P, the candidate transmission-blocking vaccine antigen from the tick, *Rhipicephalus appendiculatus*. *Int J Parasitol,* 39**,** 1485-94.

HAYES, M. J. & OLIVER, J. H., JR. 1981. Immediate and latent effects induced by the antiallatotropin precocene 2(P2) on embryonic *Dermacentor variabilis* (Say) (Acari: Ixodidae). *J Parasitol,* 67**,** 923-7.

HEEKIN, A. M., GUERRERO, F. D., BENDELE, K. G., SALDIVAR, L., SCOLES, G. A., DOWD, S. E., GONDRO, C., NENE, V., DJIKENG, A. & BRAYTON, K. A. 2013. Gut transcriptome of replete adult female cattle ticks, *Rhipicephalus (Boophilus) microplus,* feeding upon a *Babesia bovis*-infected bovine host. *Parasitol Res,* 112**,** 3075-90.

HEEKIN, A. M., GUERRERO, F. D., BENDELE, K. G., SALDIVAR, L., SCOLES, G. A., GONDRO, C., NENE, V., DJIKENG, A. & BRAYTON, K. A. 2012.

Analysis of *Babesia bovis* infection-induced gene expression changes in larvae from the cattle tick, *Rhipicephalus (Boophilus) microplus*. *Parasit Vectors,* 5:162

HEID, C. A., STEVENS, J., LIVAK, K. J. & WILLIAMS, P. M. 1996. Real time quantitative PCR. *Genome Res,* 6**,** 986-994.

HEYMAN, P., COCHEZ, C., HOFHUIS, A., VAN DER GIESSEN, J., SPRONG, H., PORTER, S. R., LOSSON, B., SAEGERMAN, C., DONOSO-MANTKE, O., NIEDRIG, M. & PAPA, A. 2010. A clear and present danger: tick-borne diseases in Europe. *Expert Review of Anti-Infective Therapy,* 8**,** 33-50.

HILBURN, L. R., DAVEY, R. B., GEORGE, J. E. & POUND, J. M. 1991. Non-random mating between *Boophilus microplus* and hybrids of *B. microplus* females and *B. annulatus* males, and its possible effect on sterile male hybrid control releases. *Exp Appl Acarol,* 11**,** 23-36.

HOFFMANN, G. 1971. Infection rates of various strains of *Boophilus* with *Babesia bigemina* and influence of host or parasite on ticks. *Zeitschrift Fur Tropenmedizin Und Parasitologie,* 22**,** 270-275.

HOMER, M. J., AGUILAR-DELFIN, I., TELFORD, S. R., KRAUSE, P. J. & PERSING, D. H. 2000. Babesiosis. *Clinical Microbiology Reviews,* 13**,** 451-469.

HOOGSTRAAL, H. 1985. Argasid and Nuttalliellid ticks as parasites and vectores. *Adv Parasit,* 24**,** 135-238.

HOVIUS, J. W. R., VAN DAM, A. P. & FIKRIG, E. 2007. Tick-host-pathogen interactions in Lyme borreliosis. *Trends Parasitol,* 23**,** 434-438.

HOYTE, H. M. 1961. Initial development of infectious *Babesia bigemina*. *Aus Vet J,* 8**,** 462-466.

HU, R. & HYLAND, K. E. 1998. Effects of the feeding process of *Ixodes scapularis* (Acari: Ixodidae) on embryonic development of its parasitoid, *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae). *J Med Entomol,* 35**,** 1050-3.

HUNFELD, K. P., HILDEBRANDT, A. & GRAY, J. S. 2008. Babesiosis: Recent insights into an ancient disease. *Int J Parasitol,* 38**,** 1219-1237.

IMAMURA, S., KONNAI, S., VAZ IDA, S., YAMADA, S., NAKAJIMA, C., ITO, Y., TAJIMA, T., YASUDA, J., SIMUUNZA, M., ONUMA, M. & OHASHI, K. 2008. Effects of anti-tick cocktail vaccine against *Rhipicephalus appendiculatus*. *JPN J Vet Res,* 56**,** 85-98.

ISEKI, H., ALHASSAN, A., OHTA, N., THEKISOE, O. M. M., YOKOYAMA, N., INOUE, N., NAMBOTA, A., YASUDA, J. & IGARASHI, I. 2007. Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine Babesia parasites. *J Microbiol Meth,* 71**,** 281-287.

JAWORSKI, D. C., SIMMEN, F. A., LAMOREAUX, W., COONS, L. B., MULLER, M. T. & NEEDHAM, G. R. 1995. A secreted calreticulin protein in Ixodid tick (*Amblyomma americanum*) saliva. *J Insect Physiol,* 41**,** 369-375.

JEMAL, A. & HUGH-JONES, M. 1993. A review of the red imported fire ant (*Solenopsis invicta Buren*) and its impacts on plant, animal, and human health. *Prev Vet Med,* 17**,** 19-32.

JENKINS, M. C. 2001. Advances and prospects for subunit vaccines against protozoa of veterinary importance. *Vet Parasitol,* 101**,** 291-310.

JOHNSTON, L. A., KEMP, D. H. & PEARSON, R. D. 1986. Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: effects of induced immunity on tick populations. *Int J Parasitol,* 16**,** 27-34.

JONGEJAN, F. & UILENBERG, G. 2004. The global importance of ticks. *Parasitology,* 129**,** S3-S14.

JONSSON, N. N., MATSCHOSS, A. L., PEPPER, P., GREEN, P. E., ALBRECHT, M. S., HUNGERFORD, J. & ANSELL, J. 2000. Evaluation of TickGARD(PLUS), a novel vaccine against *Boophilus microplus*, in lactating Holstein-Friesian cows. *Vet Parasitol,* 88**,** 275-285.

JORGENSEN, W. K., DE VOS, A. J. & DALGLIESH, R. J. 1989. Comparison of immunogenicity and virulence between *Babesia bigemina* parasites from continuous culture and from a splenectomised calf. *Aust Vet J,* 66**,** 371-2.

KAAYA, G. P., MWANGI, E. N. & OUNA, E. A. 1996. Prospects for biological control of livestock ticks, *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, using the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *J Invertebr Pathol,* 67**,** 15-20.

KAEWHOM, P., STICH, R. W., NEEDHAM, G. R. & JITTAPALAPONG, S. 2008. Molecular Analysis of Calreticulin Expressed in Salivary Glands of *Rhipicephalus (Boophilus) microplus* Indigenous to Thailand. *Animal Biodiversity and Emerging Diseases: Prediction and Prevention,* 1149**,** 53-57.

KAHL, O., GERN, L., EISEN, L. & LANE, R. S. 2002. Ecological research on *Borrelia burgdorferi* sensu lato: terminology and some methodological pitfalls. *In:* GRAY, J., KAHL, O., LANE, R. S. & STANEK, G. (eds.) *Lyme borreliosis:biology, epidemiology and control.* Wallingford, UK: CABI Publishing.

KAKOMA, I. & MEHLHORN, H. 1994. *Babesia* of domestic animals. *In:* KREIER, J. P. (ed.) *Parasitic protozoa.* London: Academic Press.

KARIM, S., MILLER, N. J., VALENZUELA, J., SAUER, J. R. & MATHER, T. N. 2005. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. *Biochem Biophys Res Commun,* 334**,** 1336-42.

KARIM, S., RAMAKRISHNAN, V. G., TUCKER, J. S., ESSENBERG, R. C. & SAUER, J. R. 2004. *Amblyomma americanum* salivary glands: double-stranded RNA-mediated gene silencing of synaptobrevin homologue and inhibition of PGE2 stimulated protein secretion. *Insect Biochem Mol Biol,* 34**,** 407-13.

KEENE, K. M., FOY, B. D., SANCHEZ-VARGAS, I., BEATY, B. J., BLAIR, C. D. & OLSON, K. E. 2004. RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *P Natl Acad Sci USA,* 101**,** 17240-17245.

KEMP, D. H., PEARSON, R. D., GOUGH, J. M. & WILLADSEN, P. 1989. Vaccination against *Boophilus microplus*: localization of antigens on tick gut cells and their interaction with the host immune system. *Exp Appl Acarol,* 7**,** 43-58.

KISS, T., CADAR, D. & SPINU, M. 2012. Tick prevention at a crossroad: New and renewed solutions. *Vet Parasitol,* 187**,** 357-366.

KLOMPEN, H. & GRIMALDI, D. 2001. First mesozoic record of a parasitiform mite: a larval argasid tick in Cretaceous amber (Acari : Ixodida : Argasidae). *Ann Entomol Soc Am,* 94**,** 10-15.

KLOMPEN, H., LEKVELSHVILI, M. & BLACK, W. C. 2007. Phylogeny of parasitiform mites (Acari) based on rRNA. *Mol Phylogen and Evol,* 43**,** 936-951.

KOCAN, K. M., BARRON, S. J., EWING, S. A. & HAIR, J. A. 1985. Transmission of *Anaplasma marginale* by adult *Dermacentor andersoni* during feeding on calves. *Am J Vet Res,* 46**,** 1565-1567.

KOCAN, K. M., DE LA FUENTE, J., BLOUIN, E. F., COETZEE, J. F. & EWING, S. A. 2010. The natural history of *Anaplasma marginale*. *Vet Parasitol,* 167**,** 95-107.

KOCAN, K. M., STILLER, D., GOFF, W. L., CLAYPOOL, P. L., EDWARDS, W., EWING, S. A., MCGUIRE, T. C., HAIR, J. A. & BARRON, S. J. 1992. Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from parasitemic to susceptible cattle. *Am J Vet Res,* 53**,** 499-507.

KOCAN, K. M., YOSHIOKA, J., SONENSHINE, D. E., DE LA FUENTE, J., CERAUL, S. M., BLOUIN, E. F. & ALMAZAN, C. 2005. Capillary tube feeding system for studying tick-pathogen interactions of *Dermacentor variabilis* (Acari : Ixodidae) and *Anaplasma marginale* (Rickettsiales : Anaplasmataceae). *J Med Entomol,* 42**,** 864-874.

KONNAI, S., YAMADA, S., IMAMURA, S., NISHIKADO, H., GITHAKA, N., ITO, T., TAKANO, A., KAWABATA, H., MURATA, S. & OHASHI, K. 2012. Identification of TROSPA homologue in *Ixodes persulcatus* Schulze, the specific vector for human Lyme borreliosis in Japan. *Ticks Tick Borne Dis,* 3**,** 75-7.

KOPACEK, P., HAJDUSEK, O. & BURESOVA, V. 2012. Tick as a Model for the Study of a Primitive Complement System. *Recent Advances on Model Hosts,* 710**,** 83-93.

KOPACEK, P., HAJDUSEK, O., BURESOVA, V. & DAFFRE, S. 2010. Tick innate immunity. *Invertebrate Immunity,* 708**,** 137-162.

KOPACEK, P., ZDYCHOVA, J., YOSHIGA, T., WEISE, C., RUDENKO, N. & LAW, J. H. 2003. Molecular cloning, expression and isolation of ferritins from two tick species - *Ornithodoros moubata* and *Ixodes ricinus*. *Insect Biochem Molec,* 33**,** 103-113.

KRANTZ, G. 1978. *A Manual of Acarology,* Salem, MA, USA, Oregon Sta. Univ. Book Stores.

KROEBER, T. & GUERIN, P. M. 2007. *In vitro* feeding assays for hard ticks. *Trends Parasitol,* 23**,** 445-449.

L'AMOREAUX, W. J., JUNAID, L. & TREVIDI, S. 2003. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. *Tissue Cell,* 35**,** 95-9.

LABUDA, M., TRIMNELL, A. R., LICKOVA, M., KAZIMIROVA, M., DAVIES, G. M., LISSINA, O., HAILS, R. S. & NUTTALL, P. A. 2006. An antivector vaccine protects against a lethal vector-borne pathogen. *Plos Pathogens,* 2**,** 251-259.

LACK, J. B., REICHARD, M. V. & VAN DEN BUSSCHE, R. A. 2012. Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. *Int J Parasitol,* 42**,** 353-363.

LATIF, A. A., BAKHEIT, M. A., MOHAMED, A. E. & ZWEYGARTH, E. 2004. High infection rates of the tick *Hyalomma anatolicum anatolicum* with *Trypanosoma theileri*. *Onderstepoort Journal of Veterinary Research,* 71**,** 251-256.

LATIF, A. A., PUTTERILL, J. F., DE KLERK, D. G., PIENAAR, R. & MANS, B. J. 2012. *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): First Description of the Male, Immature Stages and Re-Description of the Female. *Plos One,* 7.

LATIF, A. A. & WALKER, A. R. 2004. *An introduction to the biology and control of ticks in Africa,* International Consortium on Ticks and Tick Borne Disease-ICTTD.

LEW-TABOR, A. E., KURSCHEID, S., BARRERO, R., GONDRO, C., MOOLHUIJZEN, P. M., RODRIGUEZ VALLE, M., MORGAN, J. A., COVACIN, C. & BELLGARD, M. I. 2011. Gene expression evidence for off-target effects caused by RNA interference-mediated gene silencing of Ubiquitin-63E in the cattle tick *Rhipicephalus microplus*. *Int J Parasitol,* 41**,** 1001-14.

LEW-TABOR, A. E., MOOLHUIJZEN, P. M., VANCE, M. E., KURSCHEID, S., VALLE, M. R., JARRETT, S., MINCHIN, C. M., JACKSON, L. A., JONSSON, N. N., BELLGARD, M. I. & GUERRERO, F. D. 2010. Suppressive subtractive hybridization analysis of *Rhipicephalus (Boophilus) microplus* larval and adult transcript expression during attachment and feeding. *Vet Parasitol,* 167**,** 304-20.

LISBOA, R. S., TEIXEIRA, R. C., RANGEL, C. P., SANTOS, H. A., MASSARD, C. L. & FONSECA, A. H. 2009. Avian Spirochetosis in Chickens Following Experimental Transmission of *Borrelia anserina* by *Argas (Persicargas) miniatus*. *Avian Diseases,* 53**,** 166-168.

LITTLE, S. E. 2013. Future challenges for parasitology: Vector control and one health in the Americas. *Vet Parasitol,* 195**,** 249-55.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods,* 25**,** 402-408.

LIVINGSTONE, D. 1857. *Missionary travels and researches in South Africa,* London, John Murray.

LOOMIS, E. C., 1971. Rearing of *Boophilus microplus* (Acarina-Ixodidae) on laboratory rabbit. *Ann Entomol Soc Am*, 64, 598-603.

MACALUSO, K. R., MULENGA, A., SIMSER, J. A. & AZAD, A. F. 2003. Differential expression of genes in uninfected and rickettsia-infected *Dermacentor variabilis* ticks as assessed by differential-display PCR. *Infect Immun,* 71**,** 6165-70.

MACALUSO, K. R., SONENSHINE, D. E., CERAUL, S. M. & AZAD, A. F. 2001. Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* ticks acquired by artificial feeding. *Vector borne and zoonotic diseases (Larchmont, N.Y.),* 1**,** 45-53.

MACEDO, M. L., FREIRE, M., MARTINS, L. T., MARTINEZ, D. S., GOMES, V. M., SMOLKA, M. B., TOYAMA, M. H., MARANGONI, S. & COELHO, L. C. 2004. Novel protein from *Labramia bojeri* A. DC. seeds homologue to Kunitz-type trypsin inhibitor with lectin-like properties. *J Agric Food Chem,* 52**,** 7548-54.

MACKENSTEDT, U., GAUER, M., FUCHS, P., ZAPF, F., SCHEIN, E. & MEHLHORN, H. 1995. DNA measurements reveal differences in the lyfe-cycles of *Babesia bigemina* and *B. canis*, 2 typical members of the genus *Babesia*. *Parasitol Res,* 81**,** 595-604.

MAHONEY, D. F. & MIRRE, G. B. 1979. Note on the transmission of *Babesia bovis* (syn *B. argentina*) by the one-host tick *Boophilus microplus*. *Res Vet Sci,* 26**,** 253-254.

MAJOR, I. T. & CONSTABEL, C. P. 2008. Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. *Plant Physiol,* 146**,** 888-903.

MANS, B. J. 2011. Evolution of Vertebrate Hemostatic and Inflammatory Control Mechanisms in Blood-Feeding Arthropods. *J Innate Immun,* 3**,** 41-51.

MANS, B. J., DE KLERK, D., PIENAAR, R. & LATIF, A. A. 2011. *Nuttalliella namaqua*: A Living Fossil and Closest Relative the Ancestral Tick Lineage: Implications for the Evolution of Blood-Feeding in Ticks. *Plos One,* 6.

MARTINS, T. M., DO ROSARIO, V. E. & DOMINGOS, A. 2011. Identification of papain-like cysteine proteases from the bovine piroplasm *Babesia bigemina* and evolutionary relationship of piroplasms C1 family of cysteine proteases. *Exp Parasitol,* 127**,** 184-94.

MARTINS, T. M., GONCALVES, L. M., CAPELA, R., MOREIRA, R., DO ROSARIO, V. E. & DOMINGOS, A. 2010. Effect of synthesized inhibitors on babesipain-1, a new cysteine protease from the bovine piroplasm *Babesia bigemina*. *Transbound Emerg Dis,* 57**,** 68-9.

MATSUO, T., N. INOUE, M. R. RUHETA, D. TAYLOR, AND K. FUJISAKI,.2004. Tickcidal effect of monoclonal antibodies against hemocytes, Om21, in an adult female tick, *Ornithodoros moubata* (Acari : Argasidae): *J Parasitol,* 90, 715-720.

MCCOY, A. J. & KORTT, A. A. 1997. The 1.8 A crystal structure of winged bean albumin 1, the major albumin from *Psophocarpus tetragonolobus* (L.) DC. *J Mol Biol,* 269**,** 881-91.

MCHARDY, N., WOOLLON, R. M., CLAMPITT, R. B., JAMES, J. A. & CRAWLEY, R. J. 1986. Efficacy, toxicity and metabolism of imidocarb dipropionate in the treatment of *Babesia ovis* infection in sheep. *Res Vet Sci,* 41**,** 14-20.

MCNALLY, K. L., MITZEL, D. N., ANDERSON, J. M., RIBEIRO, J. M., VALENZUELA, J. G., MYERS, T. G., GODINEZ, A., WOLFINBARGER, J. B.,

BEST, S. M. & BLOOM, M. E. 2012. Differential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. *Ticks Tick Borne Dis,* 3**,** 18-26.

MEHLHORN, H. & SCHEIN, E. 1984. The piroplasms- life cycle and sexual stages. *Adv Parasit,* 23**,** 37-103.

MELLO, C. C. & CONTE, D. 2004. Revealing the world of RNA interference. *Nature,* 431**,** 338-342.

MERCADO-CURIEL, R. F., PALMER, G. H., GUERRERO, F. D. & BRAYTON, K. A. 2011. Temporal characterisation of the organ-specific *Rhipicephalus microplus* transcriptional response to *Anaplasma marginale* infection. *Int J Parasitol,* 41**,** 851-60.

MERINO, O., ALBERDI, P., PÉREZ DE LA LASTRA, J. & DE LA FUENTE, J. 2013. Tick vaccines and the control of tick-borne pathogens. *Front. Cell. Infect. Microbiol,* 3(30).

MERINO, O., ALMAZAN, C., CANALES, M., VILLAR, M., MORENO-CID, J. A., ESTRADA-PENA, A., KOCAN, K. M. & DE LA FUENTE, J. 2011a. Control of *Rhipicephalus (Boophilus) microplus* infestations by the combination of subolesin vaccination and tick autocidal control after subolesin gene knockdown in ticks fed on cattle. *Vaccine,* 29**,** 2248-54.

MERINO, O., ALMAZAN, C., CANALES, M., VILLAR, M., MORENO-CID, J. A., GALINDO, R. C. & DE LA FUENTE, J. 2011b. Targeting the tick protective antigen subolesin reduces vector infestations and pathogen infection by *Anaplasma marginale* and *Babesia bigemina*. *Vaccine,* 29**,** 8575-8579.

MICHALAK, M., CORBETT, E. F., MESAELI, N., NAKAMURA, K. & OPAS, M. 1999. Calreticulin: one protein, one gene, many functions. *Biochem Jl,* 344**,** 281-292.

MICHALAK, M., GROENENDYK, J., SZABO, E., GOLD, L. I. & OPAS, M. 2009. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem J,* 417**,** 651-66.

MIRANDA-MIRANDA, E., COSSIO-BAYUGAR, R., MARTINEZ-IBANEZ, F. & BAUTISTA-GARFIAS, C. R. 2011. Megaselia scalaris reared on *Rhipicephalus (Boophilus) microplus* laboratory cultures. *Med Vet Entomol,* 25**,** 344-7.

MIYOSHI, T., TSUJI, N., ISLAM, M. K., KAMIO, T. & FUJISAKI, K. 2004. Gene silencing of a cubilin-related serine proteinase from the hard tick *Haemaphysalis longicornis* by RNA interference. *J Vet Med Sci,* 66**,** 1471-3.

MONTEIRO, C. M., ARAUJO, L. X., MATOS, R. S., DA SILVA GOLO, P., ANGELO, I. C., DE SOUZA PERINOTTO, W. M., COELHO RODRIGUES, C. A., FURLONG, J., BITTENCOURT, V. R. & PRATA, M. C. 2013. Association between entomopathogenic nematodes and fungi for control of *Rhipicephalus microplus* (Acari: Ixodidae). *Parasitol Res*.

MONTGOMERY, M. K., XU, S. Q. & FIRE, A. 1998. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *P Natl Acad Sci USA,* 95**,** 15502-15507.

MOOLHUIJZEN, P. M., LEW-TABOR, A. E., MORGAN, J. A., VALLE, M. R., PETERSON, D. G., DOWD, S. E., GUERRERO, F. D., BELLGARD, M. I. & APPELS, R. 2011. The complexity of *Rhipicephalus (Boophilus) microplus* genome characterised. *BMC Res Notes,* 4**,** 254.

MORZARIA, S., KATENDE, J., KAIRO, A., NENE, V. & MUSOKE, A. 1992. New methods for the diagnosis of *Babesia bigemina* infection. *Mem Inst Oswaldo Cruz,* 87**,** 201-205.

MORZARIA, S. P., LATIF, A. A., JONGEJAN, F. & WALKER, A. R. 1986. Transmission of *Trypanosoma* sp. to cattle by the tick *Hyalomma anatolicum*. *Vet Parasitol,* 19**,** 13-21.

MOSQUEDA, J., OLVERA-RAMIREZ, A., AGUILAR-TIPACAMU, G. & CANTO, G. J. 2012. Current Advances in Detection and Treatment of Babesiosis. *Curr Med Chem,* 19**,** 1504-1518.

MOVILA, A., ALEKSEEV, A. N., DUBININA, H. V. & TODERAS, I. 2013. Detection of tick-borne pathogens in ticks from migratory birds in the Baltic region of Russia. *Med Vet Entomol,* 27**,** 113-117.

MULENG, A., SUGINO, M., NAKAJIM, M., SUGIMOTO, C. & ONUMA, M. 2001. Tick-Encoded serine proteinase inhibitors (serpins); potential target antigens for tick vaccine development. *J Vet Med Sci,* 63**,** 1063-9.

MULENGA, A., MACALUSO, K. R., SIMSER, J. A. & AZAD, A. F. 2003. Dynamics of Rickettsia-tick interactions: identification and characterization of differentially expressed mRNAs in uninfected and infected *Dermacentor variabilis*. *Insect Mol Biol,* 12**,** 185-93.

MURRELL, A. & BARKER, S. C. 2003. Synonymy of *Boophilus* Curtice, 1891 with *Rhipicephalus* Koch, 1844 (Acari: Ixodidae). *Syst Parasitol,* 56**,** 169-72.

MURRELL, A., CAMPBELL, N. J. & BARKER, S. C. 2000. Phylogenetic analyses of the rhipicephaline ticks indicate that the genus *Rhipicephalus* is paraphyletic. *Mol Phylogenet Evol,* 16**,** 1-7.

NARANJO, V., AYLLON, N., PEREZ DE LA LASTRA, J. M., GALINDO, R. C., KOCAN, K. M., BLOUIN, E. F., MITRA, R., ALBERDI, P., VILLAR, M. & DE LA FUENTE, J. 2013. Reciprocal Regulation of NF-kB (Relish) and Subolesin in the Tick Vector, *Ixodes scapularis*. *Plos One,* 8.

NARASIMHAN, S., MONTGOMERY, R. R., DEPONTE, K., TSCHUDI, C., MARCANTONIO, N., ANDERSON, J. F., SAUER, J. R., CAPPELLO, M., KANTOR, F. S. & FIKRIG, E. 2004. Disruption of *Ixodes scapularis* anticoagulation by using RNA interference. *Proc Natl Acad Sci U S A,* 101**,** 1141-6.

NAVA, S., GUGLIELMONE, A. A. & MANGOLD, A. J. 2009. An overview of systematics and evolution of ticks. *Frontiers in Bioscience-Landmark,* 14**,** 2857-2877.

NEEDHAM, G. R. & TEEL, P. D. 1991. Off-host physiological ecology of Ixodid ticks. *Annu Rev Entomol,* 36**,** 659-681.

NENE, V., LEE, D., KANG'A, S., SKILTON, R., SHAH, T., DE VILLIERS, E., MWAURA, S., TAYLOR, D., QUACKENBUSH, J. & BISHOP, R. 2004. Genes transcribed in the salivary glands of female *Rhipicephalus appendiculatus* ticks infected with *Theileria parva*. *Insect Biochem Mol Biol,* 34**,** 1117-28.

NIJHOF, A. M. 2010. *A contribution to the development of anti-tick vaccines.* PhD, Utrecht University.

NORIMINE, J., MOSQUEDA, J., SUAREZ, C., PALMER, G. H., MCELWAIN, T. F., MBASSA, G. & BROWN, W. C. 2003. Stimulation of T-helper cell gamma interferon and immunoglobulin G responses specific for *Babesia bovis* rhoptry-associated protein 1 (RAP-1) or a RAP-1 protein lacking the carboxy-terminal

repeat region is insufficient to provide protective immunity against virulent B-bovis challenge. *Infection and Immunity,* 71**,** 5021-5032.

NORTE, A. C., LOPES DE CARVALHO, I., RAMOS, J. A., GONCALVES, M., GERN, L. & NUNCIO, M. S. 2012. Diversity and seasonal patterns of ticks parasitizing wild birds in western Portugal. *Exp Appl Acarol,* 58**,** 327-339.

ODONGO, D., KAMAU, L., SKILTON, R., MWAURA, S., NITSCH, C., MUSOKE, A., TARACHA, E., DAUBENBERGER, C. & BISHOP, R. 2007. Vaccination of cattle with TickGARD induces cross-reactive antibodies binding to conserved linear peptides of Bm86 homologues in *Boophilus decoloratus*. *Vaccine,* 25**,** 1287-1296.

OJEDA, J. J., OROZCO, L., FLORES, R., ROJAS, C., FIGUEROA, J. V. & ALVAREZ, J. A. 2010. Validation of an attenuated live vaccine against babesiosis in native cattle in an endemic area. *Transbound Emerg Dis,* 57**,** 84-6.

OLIVER, J. H. 1989. Biology and systematics of ticks (Acari, Ixodida). *Annu Rev Ecol Syst,* 20**,** 397-430.

PAL, U., DE SILVA, A. M., MONTGOMERY, R. R., FISH, D., ANGUITA, J., ANDERSON, J. F., LOBET, Y. & FIKRIG, E. 2000. Attachment of *Borrelia burgdorferi* within *Ixodes scapularis* mediated by outer surface protein A. *Journal of Clinical Investigation,* 106**,** 561-569.

PAL, U., LI, X., WANG, T., MONTGOMERY, R. R., RAMAMOORTHI, N., DESILVA, A. M., BAO, F. K., YANG, X. F., PYPAERT, M., PRADHAN, D., KANTOR, F. S., TELFORD, S., ANDERSON, J. F. & FIKRIG, E. 2004. TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. *Cell,* 119**,** 457-468.

PARIZI, L. F., GITHAKA, N. W., LOGULLO, C., KONNAI, S., MASUDA, A., OHASHI, K. & VAZ, I. D. S., JR. 2012. The quest for a universal vaccine against ticks: Cross-immunity insights. *Vet J,* 194**,** 158-165.

PARIZI, L. F., RECH, H., SANCHEZ FERREIRA, C. A., IMAMURA, S., OHASHI, K., ONUMA, M., MASUDA, A. & VAZ, I. D. S., JR. 2009. Comparative immunogenicity of *Haemaphysalis longicornis* and *Rhipicephalus (Boophilus) microplus* calreticulins. *Vet Parasitol,* 164**,** 282-290.

PATTON, W. S. 1908. The Haemogregarines of Mammals and Reptiles. *Parasitology,* 1**,** 318-321.

PEREGRINE, A. S. 1994. Chemotherapy and delivery systems: haemoparasites. *Vet Parasitol,* 54**,** 223-48.

PERSSON, S., ROSENQUIST, M. & SOMMARIN, M. 2002. Identification of a novel calreticulin isoform (Crt2) in human and mouse. *Gene,* 297**,** 151-158.

PETER, R. J., VAN DEN BOSSCHE, P., PENZHORN, B. L. & SHARP, B. 2009. Tick, fly, and mosquito control - lessons from the past, solutions for the future. *Journal of the South African Veterinary Association-Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging,* 80**,** 121-121.

PETRIGH, R., RUYBAL, P., THOMPSON, C., NEUMANN, R., MORETTA, R., WILKOWSKY, S., DRAGHI, G., ECHAIDE, I., DE ECHAIDE, S. T. & FARBER, M. 2008. Improved Molecular Tools for Detection of *Babesia bigemina*. *Animal Biodiversity and Emerging Diseases: Prediction and Prevention,* 1149**,** 155-157.

PIPANO, E. 1995. Live vaccines against hemoparasitic diseases in livestock. *Vet Parasitol,* 57**,** 213-31.

PIRALI-KHEIRABADI, K., HADDADZADEH, H., RAZZAGHI-ABYANEH, M., BOKAIE, S., ZARE, R., GHAZAVI, M. & SHAMS-GHAHFAROKHI, M. 2007. Biological control of *Rhipicephalus (Boophilus) annulatus* by different strains of *Metarhizium anisopliae, Beauveria bassiana* and *Lecanicillium psalliotae* fungi. *Parasitol Res,* 100**,** 1297-302.

QUINELATO, S., GOLO, P. S., PERINOTTO, W. M., SA, F. A., CAMARGO, M. G., ANGELO, I. C., MORAES, A. M. & BITTENCOURT, V. R. 2012. Virulence potential of *Metarhizium anisopliae s.l.* isolates on *Rhipicephalus (Boophilus) microplus* larvae. *Vet Parasitol,* 190**,** 556-65.

RACHINSKY, A., GUERRERO, F. D. & SCOLES, G. A. 2007. Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus (Boophilus) microplus*. *Insect Biochem Molec,* 37**,** 1291-1308.

RACHINSKY, A., GUERRERO, F. D. & SCOLES, G. A. 2008. Proteomic profiling of *Rhipicephalus (Boophilus) microplus* midgut responses to infection with Babesia *bovis*. *Vet Parasitol,* 152**,** 294-313.

RAMAMOORTHI, N., NARASIMHAN, S., PAL, U., BAO, F., YANG, X. F., FISH, D., ANGUITA, J., NORGARD, M. V., KANTOR, F. S., ANDERSON, J. F., KOSKI, R. A. & FIKRIG, E. 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature,* 436**,** 573-7.

RATCLIFF, F., HARRISON, B. D. & BAULCOMBE, D. C. 1997. A similarity between viral defense and gene silencing in plants. *Science,* 276**,** 1558-1560.

RAU, U. & HANNOUN, C. 1968. Use of capillary tube technique for artificially feeding *Argas reflexus* ticks. *Bulletin of the World Health Organization,* 39**,** 332-&.

REMEDIO, R. N., SAMPIERI, B. R., VENDRAMINI, M. C., SOUZA, N. M., ANHOLETO, L. A., DENARDO, T. A. & CAMARGO-MATHIAS, M. I. 2013. Morphology of the midgut of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) adult ticks in different feeding stages. *Parasitol Res,* 112**,** 415-25.

RICKETTS, H. J. 1991. Some aspects of Rocky-mountain-spotted-feveras shownby recent investigations(reprinted from medical record, vol 76, pg 843-855, 1909). *Reviews of Infectious Diseases,* 13**,** 1227-1240.

RODRIGUEZ, M., MASSARD, C. L., DAFONSECA, A. H., RAMOS, N. F., MACHADO, H., LABARTA, V. & DE LA FUENTE, J. 1995. Effect of vaccination with a recombinant Bm86 antigen preparation on natural infestations of *Boophilus microplus* in grazing dairy and beef pure and cross bred cattle in Brazil. *Vaccine,* 13**,** 1804-1808.

RODRIGUEZ, R. I. & TREES, A. J. 1996. *In vitro* responsiveness of *Babesia bovis* to imidocarb dipropionate and the selection of a drug-adapted line. *Vet Parasitol,* 62**,** 35-41.

ROSARIO-CRUZ, R., ALMAZAN, C., MILLER, R. J., DOMINGUEZ-GARCIA, D. I., HERNANDEZ-ORTIZ, R. & DE LA FUENTE, J. 2009. Genetic basis and impact of tick acaricide resistance. *Front Biosci (Landmark Ed),* 14**,** 2657-65.

ROTH, Z., YEHEZKEL, G. & KHALAILA, I. 2012. Identification and Quantification of Protein Glycosylation. *International Journal of Carbohydrate Chemistry,* 2012.

RUDENKO, N., GOLOVCHENKO, M., EDWARDS, M. J. & GRUBHOFFER, L. 2005. Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection. *J Med Entomol,* 42**,** 36-41.

RUDZINSKA, M. A., TRAGER, W., LEWENGRUB, S. J. & GUBERT, E. 1976. Electron microscopic study of *Babesia microti* invading erythrocytes. *Cell and Tissue Research,* 169**,** 323-334.

SAITO, K. C., BECHARA, G. H., NUNES, E. T., DE OLIVEIRA, P. R., DENARDI, S. E. & MATHIAS, M. I. 2005. Morphological, histological, and ultrastructural studies of the ovary of the cattle-tick *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae). *Vet Parasitol,* 129**,** 299-311.

SAITOU, N. & NEI, M. 1986. The neighbor joining method- a new method for reconstructing phylogenetic trees. *JPN Jf Gent,* 61**,** 611-611.

SAMISH, M., GINSBERG, H. & GLAZER, I. 2004. Biological control of ticks. *Parasitology,* 129**,** S389-S403.

SAMISH, M. & REHACEK, J. 1999. Pathogens and predators of ticks and their potential in biological control. *Annu Rev Entomol,* 44**,** 159-82.

SANTOS-SILVA, M. M., BEATI, L., SANTOS, A. S., DE SOUSA, R., NUNCIO, M. S., MELO, P., SANTOS-REIS, M., FONSECA, C., FORMOSINHO, P., VILELA, C. & BACELLAR, F. 2011. The hard-tick fauna of mainland Portugal (Acari: Ixodidae): an update on geographical distribution and known associations with hosts and pathogens. *Exp Appl Acarol,* 55**,** 85-121.

SASAKI, S. D. & TANAKA, A. S. 2008. rBmTI-6, a Kunitz-BPTI domain protease inhibitor from the tick *Boophilus microplus*, its cloning, expression and biochemical characterization. *Vet Parasitol,* 155**,** 133-41.

SAUER, J. R., ESSENBERG, R. C. & BOWMAN, A. S. 2000. Salivary glands in ixodid ticks: control and mechanism of secretion. *J Insect Physiol,* 46**,** 1069-1078.

SCACHERI, P. C., ROZENBLATT-ROSEN, O., CAPLEN, N. J., WOLFSBERG, T. G., UMAYAM, L., LEE, J. C., HUGHES, C. M., SHANMUGAM, K. S., BHATTACHARJEE, A., MEYERSON, M. & COLLINS, F. S. 2004. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A,* 101**,** 1892-7.

SCHEFE, J. H., LEHMANN, K. E., BUSCHMANN, I. R., UNGER, T. & FUNKE-KAISER, H. 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's C-T difference" formula. *J Mol Med-Jmm,* 84**,** 901-910.

SCHNITTGER, L., RODRIGUEZ, A. E., FLORIN-CHRISTENSEN, M. & MORRISON, D. A. 2012. *Babesia*: A world emerging. *Infect Genet Evol,* 12**,** 1788-1809.

SCHUIJT, T. J., HOVIUS, J. W., VAN DER POLL, T., VAN DAM, A. P. & FIKRIG, E. 2011. Lyme borreliosis vaccination: the facts, the challenge, the future. *Trends Parasitol,* 27**,** 40-47.

SCHWAN, T. G. 2011. Natural History of Ticks: Evolution, Adaptation and Biology. *In:* PRESS, T. N. A. (ed.) *Critical Needs and Gaps in Understanding Prevention, Amelioration, and Resolution of Lyme and Other Tick-Borne Diseases.* The National Academies Press ed. Washington, D.C.: The National Academies Press.

SCHWAN, T. G., ANDERSON, J. M., LOPEZ, J. E., FISCHER, R. J., RAFFEL, S. J., MCCOY, B. N., SAFRONETZ, D., SOGOBA, N., MAIGA, O. & TRAORE, S. F. 2012. Endemic Foci of the Tick-Borne Relapsing Fever Spirochete *Borrelia crocidurae* in Mali, West Africa, and the Potential for Human Infection. *Plos Neglected Tropical Diseases,* 6**,** 13.

SCHWAN, T. G. & PIESMAN, J. 2002. Vector interactions and molecular adaptations of Lyme disease and relapsing fever spirochetes associated with transmission by ticks. *Emerg Infect Dis,* 8**,** 115-121.

SCHWARZ, A., VON REUMONT, B. M., ERHART, J., CHAGAS, A. C., RIBEIRO, J. M. & KOTSYFAKIS, M. 2013. De novo *Ixodes ricinus* salivary gland transcriptome analysis using two next-generation sequencing methodologies. *Faseb j*.

SHKAP, V., DE VOS, A. J., ZWEYGARTH, E. & JONGEJAN, F. 2007a. Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity. *Trends Parasitol,* 23**,** 420-426.

SHKAP, V., LEIBOVITZ, B., KRIGEL, Y., HAMMERSCHLAG, J., MARCOVICS, A., FISH, L., MOLAD, T., SAVITSKY, I. & MAZUZ, M. 2005. Vaccination of older *Bos taurus* bulls against bovine babesiosis. *Vet Parasitol,* 129**,** 235-242.

SHKAP, V., RASULOV, I., ABDURASULOV, S., FISH, L., LEIBOVITZ, B., KRIGEL, Y., MOLAD, T., MAZUZ, M. L. & SAVITSKY, I. 2007b. *Babesia bigemina*: attenuation of an Uzbek isolate for immunization of cattle with live calf- or culture-derived parasites. *Vet Parasitol,* 146**,** 221-6.

SIBLEY, L. D. 2004. Intracellular parasite invasion strategies. *Science,* 304**,** 248-253.

SILVA, F. D., REZENDE, C. A., ROSSI, D. C. P., ESTEVES, E., DYSZY, F. H., SCHREIER, S., GUEIROS-FILHO, F., CAMPOS, C. B., PIRES, J. R. & DAFFRE, S. 2009. Structure and Mode of Action of Microplusin, a Copper II-chelating Antimicrobial Peptide from the Cattle Tick *Rhipicephalus (Boophilus) microplus*. *J Biol Chem,* 284**,** 34735-46.

SILVA, M. G., DOMINGOS, A., ESTEVES, M. A., CRUZ, M. E. M. & SUAREZ, C. E. 2013. Evaluation of the growth-inhibitory effect of trifluralin analogues on *in vitro* cultured *Babesia bovis* parasites. *Int J Parasitol: Drugs and Drug Resistance,* 3**,** 59–68.

SILVA, M. G., MARQUES, P. X. & OLIVA, A. 2010a. Detection of *Babesia* and *Theileria* species infection in cattle from Portugal using a reverse line blotting method. *Vet Parasitol,* 174**,** 199-205.

SILVA, M. G., UETI, M. W., NORIMINE, J., FLORIN-CHRISTENSEN, M., BASTOS, R. G., GOFF, W. L., BROWN, W. C., OLIVA, A. & SUAREZ, C. E. 2010b. *Babesia bovis* expresses a neutralization-sensitive antigen that contains a microneme adhesive repeat (MAR) domain. *Parasitol Int,* 59**,** 294-7.

SILVA, M. M., SANTOS, A. S., FORMISINHO, P. & BACELLAR, F. 2006. Ticks associated to infectious pathologies in Portugal. *Acta medica portuguesa,* 19**,** 39-48.

SKRABALO, Z. & DEANOVIC, Z. 1957. Piroplasmosis in man; report of a case. *Documenta de medicina geographica et tropica,* 9**,** 11-6.

SMITH, A., GUO, X., DE LA FUENTE, J., NARANJO, V., KOCAN, K. M. & KAUFMAN, W. R. 2009. The impact of RNA interference of the subolesin and voraxin genes in male *Amblyomma hebraeum* (Acari: Ixodidae) on female engorgement and oviposition. *Exp Appl Acarol,* 47**,** 71-86.

SMITH, T. & KILBORNE, F. L. 1893. Investigations into the nature, causation, and prevention of Texas or southern cattle fever. *Bureau of Animal Industries.* Washington D.C.: U.S. Department of Agriculture.

SOARES, C. A., LIMA, C. M., DOLAN, M. C., PIESMAN, J., BEARD, C. B. & ZEIDNER, N. S. 2005. Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol,* 14**,** 443-52.

SONENSHINE, D. E. 1991. *Biology of Ticks,* New York, Oxford University Press.

SONENSHINE, D. E., CERAUL, S. M., HYNES, W. E., MACALUSO, K. R. & AZAD, A. F. 2002. Expression of defensin-like peptides in tick hemolymph and midgut in response to challenge with *Borrelia burgdorferi*, *Escherichia coli* and *Bacillus subtilis. Exp Appl Acarol,* 28**,** 127-134.

STAFFORD, K. C., 3RD & ALLAN, S. A. 2010. Field applications of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* F52 (Hypocreales: Clavicipitaceae) for the control of *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol,* 47**,** 1107-15.

SUAREZ, C. E. & NOH, S. 2011. Emerging perspectives in the research of bovine babesiosis and anaplasmosis. *Vet Parasitol,* 180**,** 109-125.

SUAREZ, C. E., PALMER, G. H., FLORIN-CHRISTENSEN, M., HINES, S. A., HOTZEL, I. & MCELWAIN, T. F. 2003. Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina* rap-1 locus. *Mol Biochem Parasit,* 127**,** 101-112.

SUKUMARAN, B., NARASIMHAN, S., ANDERSON, J. F., DEPONTE, K., MARCANTONIO, N., KRISHNAN, M. N., FISH, D., TELFORD, S. R., KANTOR, F. S. & FIKRIG, E. 2006. An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. *J Exp Med,* 203**,** 1507-17.

TAYLOR, R. J. & MCHARDY, N. 1979. Preliminary observations on the combined use of imidocarb and *Babesia* blood vaccine in cattle. *J S Afr Vet Assoc,* 50**,** 326-9.

TORINA, A., AGNONE, A., SIRECI, G., MOSQUEDA, J. J., BLANDA, V., ALBANESE, I., LA FARINA, M., CERRONE, A., CUSUMANO, F. & CARACAPPA, S. 2010. Characterization of the apical membrane antigen-1 in Italian strains of *Babesia bigemina*. *Transbound Emerg Dis,* 57**,** 52-6.

TRAGER, W. 1939. Further observations on acquired immunity to tick *Dermacentor variabilis,* Say. *J Parasitol,* 25**,** 137-139.

TRIMNELL, A. R., DAVIES, G. M., LISSINA, O., HAILS, R. S. & NUTTALL, P. A. 2005. A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. *Vaccine,* 23**,** 4329-41.

TRIMNELL, A. R., HAILS, R. S. & NUTTALL, P. A. 2002. Dual action ectoparasite vaccine targeting 'exposed' and 'concealed' antigens. *Vaccine,* 20**,** 3560-8.

TSUJI, N., BATTSETSEG, B., BOLDBAATAR, D., MIYOSHI, T., XUAN, X., OLIVER, J. H., JR. & FUJISAKI, K. 2007. Babesial vector tick defensin against *Babesia* sp. parasites. *Infect Immun,* 75**,** 3633-40.

TSUJI, N., MIYOSHI, T., BATTSETSEG, B., MATSUO, T., XUAN, X. & FUJISAKI, K. 2008. A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *Plos Pathogens,* 4.

TURELL, M. 1988. Horizontal and vertical transmission of viruses by insect and tick vectors. *In:* MONATH, T. (ed.) *The Arboviruses: Ecology and Epidemiology.* Boca Raton: CRC Press.

UILENBERG, G. 2006. *Babesia* - A historical overview. *Vet Parasitol,* 138**,** 3-10.

UNIVERSITY, P. 2013. *Public Health and Medical Entomology | Purdue | Biology | Entomology | Insects | Ticks | Diseases | Monitoring | Control | Hot Topics | Agriculture | Extension* [Online]. Available: http://extension.entm.purdue.edu/publichealth/print/insects/tick.html. [Accessed 27th July 2013]

URIELI-SHOVAL, S., LINKE, R. P. & MATZNER, Y. 2000. Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. *Curr Opin Hematol,* 7**,** 64-9.

URQUHART, G. M., ARMOUR, J., DUNCAN, J. L., JENNINGS, F. W. & A.M., D. 1996. *Vet Parasitol,* Oxford, United Kingdom, Blackwell Science.

VALLE, M. R., MENDEZ, L., VALDEZ, M., REDONDO, M., ESPINOSA, C. M., VARGAS, M., CRUZ, R. L., BARRIOS, H. P., SEOANE, G., RAMIREZ, E. S., BOUE, O., VIGIL, J. L., MACHADO, H., NORDELO, C. B. & PINEIRO, M. J. 2004. Integrated control of *Boophilus microplus* ticks in Cuba based on vaccination with the anti-tick vaccine Gavac (TM). *Exp Appl Acarol,* 34**,** 375-382.

VEIGA, L. P., SOUZA, A. P., BELLATO, V., SARTOR, A. A., NUNES, A. P. & CARDOSO, H. M. 2012. Resistance to cypermethrin and amitraz in *Rhipicephalus (Boophilus) microplus* on the Santa Catarina Plateau, Brazil. *Rev Bras Parasitol Vet,* 21**,** 133-6.

VIAL, H. J. & GORENFLOT, A. 2006. Chemotherapy against babesiosis. *Vet Parasitol,* 138**,** 147-60.

VILLAR, M., TORINA, A., NUNEZ, Y., ZIVKOVIC, Z., MARINA, A., ALONGI, A., SCIMECA, S., LA BARBERA, G., CARACAPPA, S., VAZQUEZ, J. & FUENTE JDE, L. 2010. Application of highly sensitive saturation labeling to the

analysis of differential protein expression in infected ticks from limited samples. *Proteome Sci,* 8**,** 43.

VU HAI, V., PAGES, F., BOULANGER, N., AUDEBERT, S., PAROLA, P. & ALMERAS, L. 2013. Immunoproteomic identification of antigenic salivary biomarkers detected by. *Ticks Tick Borne Dis,* 4**,** 459-468.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WASER, M., MESAELI, N., SPENCER, C. & MICHALAK, M. 1997. Regulation of calreticulin gene expression by calcium. *J Cell Biol,* 138**,** 547-557.

WEIGL, B., DOMINGO, G., LABARRE, P. & GERLACH, J. 2008. Towards non- and minimally instrumented, microfluidics-based diagnostic devices. *Lab Chip,* 8**,** 1999-2014.

WILLADSEN, P. 2004. Anti-tick vaccines. *Parasitology,* 129**,** S367-S387.

WILLADSEN, P. 2006. Tick control: Thoughts on a research agenda. *Vet Parasitol,* 138**,** 161-168.

WILLADSEN, P. 2008. Antigen cocktails: valid hypothesis or unsubstantiated hope? *Trends Parasitol,* 24**,** 164-167.

WILLADSEN, P. & JONGEJAN, F. 1999. Immunology of the tick-host interaction and the control of ticks and tick-borne diseases. *Parasitol Today,* 15**,** 258-262.

WILLADSEN, P. & KEMP, D. H. 1988. Vaccination with concealed antigens for tick control. *Parasitol Today,* 4**,** 196-198.

WILLADSEN, P., RIDING, G. A., MCKENNA, R. V., KEMP, D. H., TELLAM, R. L., NIELSEN, J. N., LAHNSTEIN, J., COBON, G. S. & GOUGH, J. M. 1989. Immunological control of a parasitic arthropod – identification of a protective antigen from *Boophilus microplus*. *J Immunol,* 143**,** 1346-1351.

XU, G. A., FANG, Q. Q., KEIRANS, J. E. & DURDEN, L. A. 2004. Cloning and sequencing of putative calreticulin complementary DNAs from four hard tick species. *J Parasitol,* 90**,** 73-78.

ZHU, Z. 1998. Histological observations on *Borrelia burgdorferi* growth in naturally infected female *Ixodes ricinus*. *Acarologia,* 39**,** 11-22.

ZINTL, A., GRAY, J. S., SKERRETT, H. E. & MULCAHY, G. 2005. Possible mechanisms underlying age-related resistance to bovine babesiosis. *Parasite Immunol,* 27**,** 115-120.

ZIVKOVIC, Z., ESTEVES, E., ALMAZAN, C., DAFFRE, S., NIJHOF, A. M., KOCAN, K. M., JONGEJAN, F. & DE LA FUENTE, J. 2010a. Differential expression of genes in salivary glands of male *Rhipicephalus (Boophilus)microplus* in response to infection with *Anaplasma marginale*. *Bmc Genomics,* 11**,** 12.

ZIVKOVIC, Z., TORINA, A., MITRA, R., ALONGI, A., SCIMECA, S., KOCAN, K. M., GALINDO, R. C., ALMAZAN, C., BLOUIN, E. F., VILLAR, M., NIJHOF, A. M., MANI, R., LA BARBERA, G., CARACAPPA, S., JONGEJAN, F. & DE LA FUENTE, J. 2010b. Subolesin expression in response to pathogen infection in ticks. *Bmc Immunology,* 11**,** 12.

ZUCKERKANDL, E. & PAULING, L. 1965. *Evolutionary Divergence and Convergence in Proteins,* New York, Academic Press.

WEBSITE: The History of Animals.” Book V part 31. Translated by D'Arcy Wentworth Thompson : http://classics.mit.edu//Aristotle/history_anim.html.

Appendix

Appendix 1

Table 1. Candidate differentially expressed genes in *R. annulatus* ticks in response to *B. bigemina* infection.

EST	Genbank accession number	Length	Percent AT	Poly A at	Reverse ?	Contig Number	Number of Sequences	Comments
RA-EST_1	JK489362	943	39,45		N	1	2	cuticle protein [Brevicoryne brassicae]
RA-EST_2	JK489363	501	38,12		N	2	5	cuticle protein
RA-EST_3	JK489364	663	37,56		N	3	2	cuticle protein
RA-EST_4	JK489365	1135	37,71		N	4	38	structural molecule
RA-EST_5	JK489366	550	46,55		N	5	9	yolk cathepsin
RA-EST_6	JK489367	670	40,90		N	6	74	yolk cathepsin
RA-EST_7	JK489368	543	48,80		N	7	14	glutaminyl-tRNA synthetase
RA-EST_8	JK489369	879	57,57		N	8	4	putative salivary protein
RA-EST_9	JK489370	862	53,36		N	9	4	Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11 isoform 4
RA-EST_10	JK489371	599	63,44		N	10	2	Unknown
RA-EST_11	JK489372	502	67,93		N	11	8	Unknown
RA-EST_12	JK489373	803	70,11		N	12	160	Unknown
RA-EST_13	JK489374	658	50,00		N	13	1	hebreain-like
RA-EST_14	JK489375	1066	50,84		N	14	6	LOC512799 protein, Cofactor of BRCA1 (COBRA1) [PREDICTED: Bos taurus mRNA]
RA-EST_15	JK489376	579	50,09		N	15	28	hebreain-like
RA-EST_16	JK489377	753	40,11		N	16	2	Unknown
RA-EST_17	JK489378	1011	40,16		N	17	42	Salivary gland secretion 1 CG3047-PA
RA-EST_18	JK489379	429	47,09		N	18	4	Unknown
RA-EST_19	JK489380	281	50,18		N	19	8	Unknown
RA-EST_20	JK489381	607	50,08		N	20	10	Unknown
RA-EST_21	JK489382	552	43,30		N	21	14	Calreticulin
RA-EST_22	JK489383	353	43,06		N	22	6	hupothetical methyltransferase protein [PREDICTED: Bos taurus mRNA]
RA-EST_23	JK489384	825	44,61		N	23	1	DnaJ (Hsp40) homolog, subfamily C, member 3
RA-EST_24	JK489385	277	46,21		N	24	1	Kunitz-type proteinase inhibitor SHPI-1 Chain, Trypsin Inhibitor
RA-EST_25	JK489386	286	51,75		N	25	1	Kunitz-type protease inhibitor 5 II (SA5 II)
RA-EST_26	JK489387	408	43,38		N	26	4	Protease inhibitor carrapatin
RA-EST_27	JK489388	374	41,98		N	27	2	Protease inhibitor carrapatin
RA-EST_28	JK489389	818	46,58		N	28	8	Kunitz-like serine protease inhibitor
RA-EST_29	JK489390	1657	46,17		N	29	11	Kunitz-like protease inhibitor precursor
RA-EST_30	JK489391	528	52,84		N	30	2	hypothetical protein BBOV_II006290 [PREDICTED: Babesia mRNA]
RA-EST_31	JK489392	520	49,04		N	31	4	Unknown
RA-EST_32	JK489393	181	42,54		N	32	6	Unknown
RA-EST_33	JK489394	514	49,42		N	33	9	microplusin-like antimicrobial
RA-EST_34	JK489395	504	47,22		N	34	4	Unknown
RA-EST_35	JK489396	667	39,58		N	35	6	GP80 precursor

RA-EST_36	JK489397	175	38,29		N	36	21	GP80 precursor
RA-EST_37	JK489398	550	55,45		N	37	2	Unknown
RA-EST_38	JK489399	553	47,74		N	38	2	microplusin preprotein
RA-EST_39	JK489400	471	40,13		N	39	6	FKBP-type peptidyl-prolyl cis-trans isomerase
RA-EST_40	JK489401	254	43,31		N	40	4	Unknown
RA-EST_41	JK489402	620	52,74		N	41	6	40S ribosomal protein S3a
RA-EST_42	JK489403	341	53,67	1	Y	42	6	Kunitz-type protease inhibitor 5 II (SA5 II)
RA-EST_43	JK489404	393	43,26		N	43	4	Unknown
RA-EST_44	JK489405	404	50,74		N	44	10	Unknown
RA-EST_45	JK489406	1394	51,87		N	45	12	aspartic protéase
RA-EST_46	JK489407	416	56,49		N	46	8	endonuclease/reverse transcriptase [PREDICTED: Bos taurus mRNA]
RA-EST_47	JK489408	415	53,49	1	Y	47	2	Unknown
RA-EST_48	JK489409	627	51,99		N	48	8	Unknown
RA-EST_49	JK489410	478	50,84		N	49	2	Similar to reticulon 4 interacting protein 1
RA-EST_50	JK489411	447	55,03		N	50	4	Unknown
RA-EST_51	JK489412	423	43,97		N	51	2	Endothelin-converting enzyme
RA-EST_52	JK489413	216	38,43		N	52	4	Unknown
RA-EST_53	JK489414	629	48,97		N	53	4	Unknown
RA-EST_54	JK489415	362	56,91		N	54	6	Unknown
RA-EST_55	JK489416	333	55,26		N	55	4	Unknown
RA-EST_56	JK489417	541	60,44	522	N	56	3	Unknown
RA-EST_57	JK489418	482	53,11		N	57	6	methionine adenosyltransferase [PREDICTED: Bos taurus mRNA]
RA-EST_58	JK489419	105	69,52	86	N	58	6	Unknown
RA-EST_59	JK489420	955	56,34		N	59	2	Unknown
RA-EST_60	JK489421	1646	46,29		N	60	6	chaperonin [Aedes aegypti]
RA-EST_61	JK489422	276	56,52	1	Y	61	2	GP80 precursor
RA-EST_62	JK489423	297	45,12		N	62	2	von Willebrand factor
RA-EST_63	JK489424	993	51,56		N	63	2	similar to CG12070-PA, isoform A isoform 1
RA-EST_64	JK489425	893	45,24		N	64	5	similar to GA12600-PA isoform 1
RA-EST_65	JK489426	355	55,49	336	N	65	4	similar to Proteasome subunit beta type 1 (Proteasome 26 kDa subunit)
RA-EST_66	JK489427	496	48,79		N	66	2	similar to CG6672-PA, partial
RA-EST_67	JK489428	645	52,71		N	67	1	similar to CG10584-PA
RA-EST_68	JK489429	496	36,29		N	68	1	TROSPA
RA-EST_69	JK489430	359	54,87	340	N	69	7	Unknown
RA-EST_70	JK489431	448	54,24		N	70	2	Unknown
RA-EST_71	JK489432	256	58,20		N	71	6	Unknown
RA-EST_72	JK489433	802	57,86		N	72	4	RE37250p Transcription factor
RA-EST_73	JK489434	536	48,32		N	73	2	keratin 2 (epidermal ichthyosis bullosa of Siemens)
RA-EST_74	JK489435	517	53,19		N	74	4	Unknown
RA-EST_75	JK489436	415	52,29		N	75	4	Unknown
RA-EST_76	JK489437	210	37,62		N	76	4	Unknown
RA-EST_77	JK489438	438	56,85		N	77	4	Unknown
RA-EST_78	JK489439	511	49,12		N	78	4	Unknown
RA-EST_79	JK489440	288	45,83		N	79	3	Unknown
RA-EST_80	JK489441	611	52,86		N	80	7	Unknown

RA-EST_81	JK489442	324	41,67		N	81	8	serum amyloid A protein-like
RA-EST_82	JK489443	307	50,49		N	82	2	Unknown
RA-EST_83	JK489444	718	48,19		N	83	2	similar to 5-3 exoribonuclease 1 [Tribolium castaneum]
RA-EST_84	JK489445	385	42,08		N	84	3	Ricinusin
RA-EST_85	JK489446	356	57,30		N	85	1	Unknown
RA-EST_86	JK489447	277	58,84		N	86	2	Unknown
RA-EST_87	JK489448	894	46,87		N	87	1	hypothetical protein
RA-EST_88	JK489449	424	53,54	405	N	88	4	Unknown
RA-EST_89	JK489450	1052	49,81		N	89	2	Unknown
RA-EST_90	JK489451	519	48,36		N	90	4	Unknown
RA-EST_91	JK489452	366	48,36	1	Y	91	2	F1F0-type ATP synthase subunit g
RA-EST_92	JK489453	173	44,51		N	92	1	yolk cathepsin
RA-EST_93	JK489454	1113	50,31		N	93	2	similar to CG3999-PA
RA-EST_94	JK489455	236	42,80		N	94	2	similar to CG3214-PA
RA-EST_95	JK489456	401	52,87		N	95	2	FSHD region gene 1 [PREDICTED: Bos taurus mRNA]
RA-EST_96	JK489457	872	40,02		N	96	2	Unknown

Appendix 2

```
CNNNCNNNNTCGCTATATCGGCCGACCCGACCGTATACTTCAAAGAGGAGTTCA
ACGATGGAGACGCGTGGAAGGACCGGTGGGTGGAGTCTACGAAAGGCGACAAC
CTCGGAAAGTTCGTTCTAAGCGCTGGCAAGTTCTACGGTGACGCGGAGAAGAGC
AAAGGACTGCAGACCTCTGAAGACGCCCGCTTCTACGGCATCTCCGCCAAGTTCG
AACCCTTCTCCAACGAAGACAAGACCCTGGTCATCCAATTCGCGGTGAAGCACG
AGCAGAACATCGACTGCGGCGGTGGCTACGTCAAGCTGTTCGACTGCAGCCTAG
ACCAGACTCAAATGCACGGCGAATCTCCCTACCTCATCATGTTCGGCCCTGACAT
CTGCGGTCCCGGCACCAAAAAAGTGCACGCCATCTTCAACTACAAGGGCAAGAA
CCACCTTATCAACAAGGAGGTGCGCTGCAAAGACGACGTCTTCAGCCACCTGTAC
ACCCTGATCGTTAAGCCCGATAACACATACCAGATCAAGATCGACAACGAAGTG
GTCGAGAAGGGCGAACTCGAGAAGGACTGGTCCTTCCTGCCCCCCAAGAAGATC
AAGGACCCCGACGCCAAGAAGCCCGAGGACTGGGACGACCGGGCCAAGATCGA
CGACCCCGACGACAAGAAGCCCGAGGACTGGGACAAGCCCGAGTACATTCCCGA
CCCGGACGCCACCAAGCCCGAGGACTGGGACGACGACATGGACGGCGAGTGGG
AACCCCCGCAGATCAACAACCCCGAGTACAAGGGCGAGTGGAAACCCAAGCAG
ATCGACAACCCGGCCTACAAGGGTGCCTGGGTACACCCGGAGATCGACAACCCC
GAGTACACGCCGGACCCCAAGCTGTACCGCTACAAGGAGATCTGCAAGATCGGC
TTCGACCTGTGGCAGGTCAAGTCTGGCACCATCTTCGACAACATCCTCATCACGG
ACGACGAAGAGTACGCCCGGGTGCACGGCGAGGAGACCTGGGCCGCGCTCAAN
ACGAGAGAAAAGATGANNNCAAGCAGGAAGAGAGAGAGCAAAGAGCAGAAGA
GATGACGCTAGACGAGGACGANN
```

Figure 38: Sequencing of obtained fragment from CRT coding region amplification.

```
B.taurus          MLLPVPLLLGLLGLAAADPTVYFKEQFLDGDGWTERWIESKHKPDFGKFVLSSGKFYGDQ
O.cuniculus       MLLPVPLLLGLLGLAAAEPVVYFKEQFLDGDGWTERWIESKHKSDFGKFVLSSGKFYGDQ
M.musculus        MLLSVPLLLGLLGLAAADPAIYFKEQFLDGDAWTNRWVESKHKSDFGKFVLSSGKFYGDL
R.annulatus       -MRLLCILLPLVGLISADPTVYFKEEFNDGDAWKDRWVESTKGDNLGKFVLSAGKFYGDA
R.microplus       -MRLLCILLPLVGLISADPTVYFKEEFNDGDAWKDRWVESTKGDNLGKFVLSAGKFYGDA
                   :  : :** *:** :*:*.:****:* ***.*.:**:**.:  ::******:******
B.taurus          EKDKGLQTSQDARFYALSARFEPFSNKGQTLVVQFTVKHEQNIDCGGGYVKLFPAGLDQT
O.cuniculus       EKDKGLQTSQDARFYALSARFEPFSNKGQPLVVQFTVKHEQNIDCGGGYVKLFPAGLDQK
M.musculus        EKDKGLQTSQDARFYALSAKFEPFSNKGQTLVVQFTVKHEQNIDCGGGYVKLFPSGLDQK
R.annulatus       EKSKGLQTSEDARFYGISAKFEPFSNEDKTLVIQFTVKHEQNIDCGGGYVKLFDCSLDQT
R.microplus       EKSKGLQTSEDAHFYGISAKFEPFSNEDKTLVIQFTVKHEQNIDCGGGYVKLFDCSLDQT
                  **.******:**:**.:**:******:.:.**:********************* ..***.
B.taurus          DMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVRPNN
O.cuniculus       DMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVRPDN
M.musculus        DMHGDSEYNIMFGPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVRPDN
R.annulatus       QMHGESPYLIMFGPDICGPGTKKVHAIFNYKGKNHLINQEVRCKDDVFSHLYTLIVKPDN
R.microplus       QMHGESPYLIMFGPDICGPGTKKVHAIFNYKGKNHLINKEVRCKDDVFSHLYTLIVKPDN
                  :***:* * ****************.******** ***:::***** *:*******:*:*
B.taurus          TYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDDRAKIDDPTDSKPEDWDKPE
O.cuniculus       TYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDKPE
M.musculus        TYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDAAKPEDWDERAKIDDPTDSKPEDWDKPE
R.annulatus       TYQIKIDNEVVEKGELEKDWSFLPPKKIKDPDAKKPEDWDDRAKIDDPDDKKPEDWDKPE
R.microplus       TYQIKIDNEVVEKGELEKDWSFLPPKKIKDPDAKKPEDWDDRAKIDDPDDKKPEDWDKPE
                  **::****. **.*.**.**.************ ******:******* *.*********
B.taurus          HIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPEYKGIWIHPEIDNPEYS
O.cuniculus       HIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWIHPEIDNPEYS
M.musculus        HIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWIHPEIDNPEYS
R.annulatus       YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
R.microplus       YIPDPDATKPEDWDDDMDGEWEPPQINNPEYKGEWKPKQIDNPAYKGAWVHPEIDNPEYT
                  :******.******::******** *:**********:***** *** *:**********:
B.taurus          PDSNIYAYENFAVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKAAEKQMKDK
O.cuniculus       PDANIYAYDSFAVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKTAEKQMKDK
M.musculus        PDANIYAYDSFAVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKAAEKQMKDK
R.annulatus       PDPKLYRYKEICKIGFDLWQVKSGTIFDNILITDDEEYARVHGEETWAALKDEEKKMKDK
R.microplus       PDPKLYRYKEICKIGFDLWQVKSGTIFDNILITDDEEYARVHGEETWAALKDEEKKMKDK
                  **.::* *..:. :*:**************:***:** **. .*:***.. *  **:****
B.taurus          QDEEQRLHEEEEEKKGKEEEEADKDDDEDKDEDEEDEDEKE-EEEEEDAAAGQAKDEL
O.cuniculus       QDEEQRLKEEEEEKKRKEEEEAEEDEEDKDDKEDEDEDEEDKDEEEEEAAAGQAKDEL
M.musculus        QDEEQRLKEEEEDKKRKEEEEAE--DKEDDDDRDEDEDEEDEKEEDEEESPGQAKDEL
R.annulatus       QEEEEEAKSKKEDDAKDEDEFEDDEDKDEDKKDEEETTPAPEDDEDHKHEEL------
R.microplus       QEEEEEAKSKKDDDAKDEDEFEDDEDKDEDKKDEEETTPAPEDDEDHKHEEL------
                  *:**:. :.:::::.  .*:*  :  :.:.... :*:      .:*:..
```

Figure 39: Alignment of available calreticulin protein sequences,
Rhipicephalus annulatus (accession number AAR29939), *R. microplus* (AGK88372), *Oryctolagus cuniculus* (AAA31188), *Mus musculus* (AAH03453) and *Bos taurus* (BAB86913) using Clustal W. asterisks denote conserved amino acids.

Appendix 3

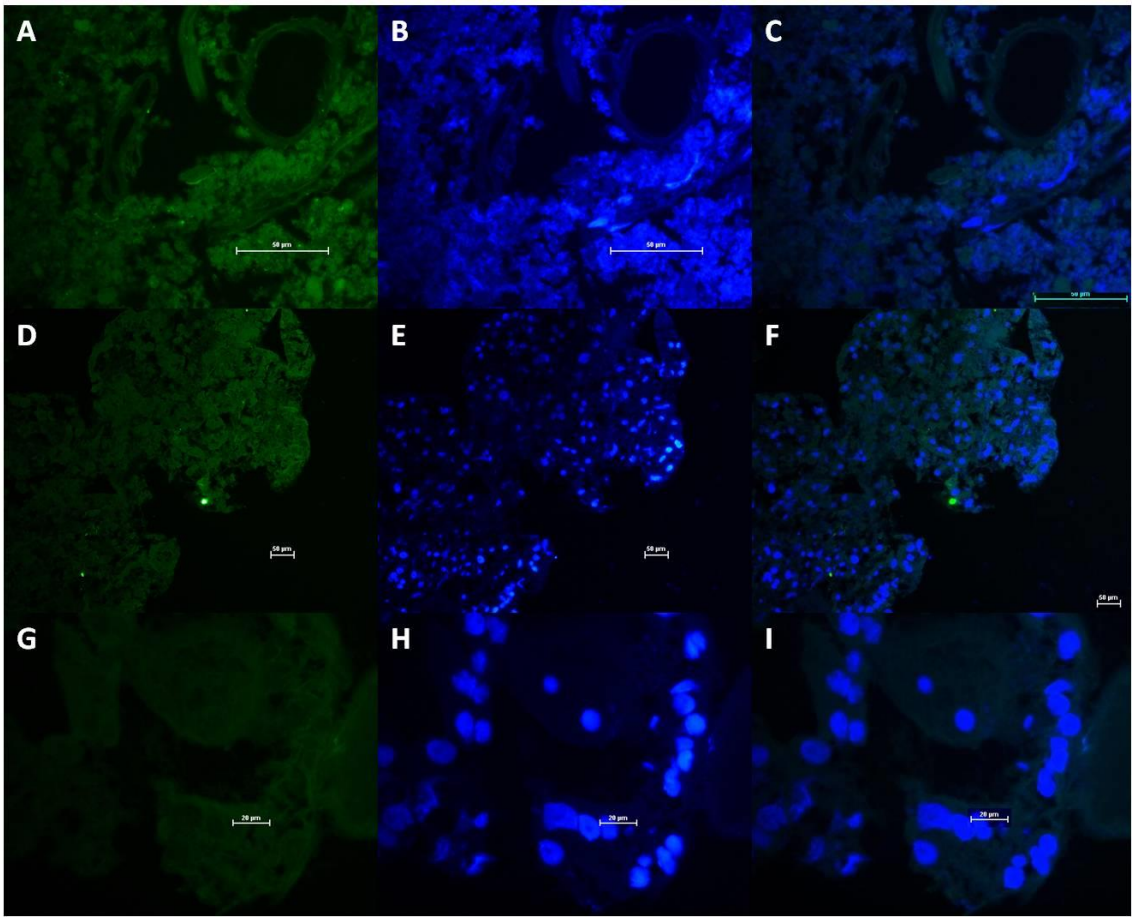

Figure 40: Negative control of immunofluorescence analysis of cattle tick tissues.
Sections of 2-3 µm of different tick tissues were made and subsequently incubated with PBS. Anti-mouse Alexa Green 488 (Invitrogen) conjugated was used as secondary antibody and slides were visualized under a Nikon fluorescence microscope. Nuclear DNA was stained with DAPI. (A) Salivary gland section under a fluorescein filter at 40X amplification. B) Salivary gland section with Dapi staining. (C) Merge of both staining in salivary gland. (D) Midgut section under a fluorescein filter at 10X amplification. (E) Midgut section with DAPI staining. (F) Merge of both staining in midgut section. (G) Ovaries section under a fluorescein filter at 40X amplification. B) Ovaries section with DAPI staining. (C) Merge of both staining in ovaries section. Bars, 50 µm

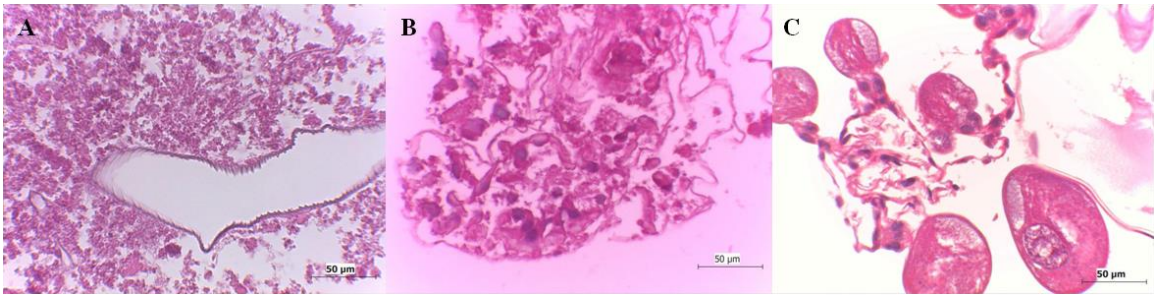

Figure 41: Microscopic view of a histological specimen of *Rhipicephalus annulatus* tick tissues stained with hematoxylin and eosin.
(A) Salivary glands; (B) Midgut; (C) Ovary; Bars 50µm (*original from the author*)